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Ribonucleotide reductase and SAMHD1:

Critical players in nucleotide metabolism, NRTI efficacy and HIV replication

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B.S., State University of NY Oneonta, 2011

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Abstract

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By Michele Daly

2' Deoxyribonucleotides (dNTPs) are essential for the DNA replication of all organisms and many viruses. Cellular dNTP regulation is an exquisitely complex process, which includes two major enzymes: ribonucleotide reductase (RNR) and sterile alpha motif and histidine-aspartic domain containing protein 1 (SAMHD1). RNR is responsible for the *de novo* synthesis of dNTPs through the reduction of ribonucleotides. Reciprocally, SAMHD1 is responsible for the hydrolysis of dNTPs to dNs and triphosphates. These two enzymes, working in concert, maintain cellular dNTP concentrations at the proper levels for genomic replication and DNA repair. Even modest disruptions in the specific balance of dNTPs can lead to poor DNA polymerase fidelity and proofreading, resulting in genome instability and mutagenesis.

Human immunodeficiency virus (HIV) is a significant human pathogen with approximately 36.9 million people infected worldwide. Without treatment, chronic HIV infection depletes CD4 lymphocytes, which are necessary for maintaining immunocompetence, leading to acquired immunodeficiency syndrome (AIDS). HIV replication occurs via reverse transcription of the viral RNA genome to DNA by the viral polymerase, reverse transcriptase (RT). This process, which requires cellular dNTPs, has been clinically exploited with nucleoside reverse transcriptase inhibitors (NRTIs). NRTIs are structurally analogous to dNTPs, and due to the low fidelity of RT they are readily incorporated during viral replication. All seven of the FDA-approved NRTIs induce obligate chain termination, because unlike dNTPs they do not have the chemical requirements to make a bond with the next incoming dNTP.

Therapeutic innovation is essential due to the increased prevalence of NRTI resistance, transmission of drug resistant variants and necessity of salvage therapy. Here, we investigate multiple NRTIs, which are varied in their mechanism of action, and the role that RNR and SAMHD1 have in modulating their antiviral activity. First, we investigated the purine analog clofarabine, an FDA approved anticancer compound. Clofarabine works via two mechanisms. It inhibits RNR causing a decrease in cellular dNTPs and its incorporation by RT induces delayed chain termination. RNR inhibition limits competition for the incorporation of clofarabine by RT and its antiviral activity is self-potentiated. Second, we examined the anti-HIV mechanism of 2 FDA-approved lethal mutagens, 5-azacytidine and 5-aza-2'deoxycytidine. Our results show that RNR rapidly reduces 5-azacytidine to 5-aza-2'deoxycytidine, and therefore the active drug is the deoxyribose form. Lastly, we studied how SAMHD1 induced cellular dNTP depletion affects the competitive landscape for NRTIs. As expected, SAMHD1 dNTP depletion increased NRTI efficacy. Importantly SAMHD1 does not effectively degrade the clinically relevant NRTIs. Taken together, our results indicate that novel NRTIs, whether they are an obligate chain terminator, delayed chain terminator, or lethal mutagen should be screened for the following characteristics: 1) Inhibition of RNR, 2) Inhibition of HIV-RT, including NRTI resistant variants and 3) Resistance to degradation by SAMHD1.

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I would like to dedicate this dissertation to my parents. My father's insatiable thirst for knowledge has always been an inspiration for me to continue learning. My mother's laissez-faire parenting style has allowed me the freedom and confidence to pursue my dreams. Without their love and support, I would not have had this opportunity. Words cannot describe how much I love you both and I am eternally grateful for all you have done for me.

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LIST OF ABBREVIATIONS

3TC: Lamivudine
AGS: Aicardi-Goutières syndrome
AIDS: Acquired immunodeficiency syndrome
ABC: Abacavir
ARV: Antiretroviral
AZT: Azidothymidine
Capsid: CA
cPPT: Central polypurine tract
d4T: Stavudine
ddI: Didanosine
dNDP: deoxynucleotide diphosphate
dNTP: deoxynucleotide triphosphate
Env: Envelope
FTC: Emtricitabine
Gag: Group specific antigen
Gp120/41: glycoprotein120/41
HAART: Highly active antiretroviral therapy
HAND: HIV-1 associated neurocognitive disorder
HIV-1: Human immunodeficiency virus type 1
HIV-2: Human immunodeficiency virus type 2
IN: Integrase
INSTI: Integrase strand transfer inhibitor
MA: Matrix
NDP: Ribonucleoside diphosphate
NTP: Ribonucleoside triphosphate
Nef: Negative regulatory factor
NNRTI: Non-nucleoside reverse transcriptase inhibitor
NRTI: Nucleoside reverse transcriptase inhibitor
NC: Nucleocapsid
ORF: Open reading frame
PBS: Primer binding site
PI: Protease inhibitor
PIC: Pre-integration complex
Pol: Polymerase
PPT: Polypurine tract
PR: Protease
Rev: Regulator of expression of viral proteins
RT: Reverse transcriptase:
RNR: Ribonucleotide reductase
SAM: sterile alpha motif
SAMHD1: Sterile alpha motif and histidine-aspartic domain containing protein 1
SIV: Simian immunodeficiency virus
TAMs: Thymidine analog mutations
Tat: Trans-activator of transcription
TDF: Tenofovir disproxil fumarate
Vif: viral infectivity factor
Vpr/Vpu/Vpx: Viral protein r/u/x

CHAPTER I

(A) HIV/AIDS

(a) Origins

Acquired immunodeficiency syndrome (AIDS) is a chronic human disease that causes a dramatic decline in CD4+ T lymphocytes. CD4+ T cells are essential for maintaining immunocompetence, and when they reach critically low levels patients are susceptible to opportunistic infections and neoplasms. In 1980-1981, there were reports of previously healthy young homosexual men presenting with opportunistic infections such as Cytomegalovirus, *Pneumocystis carinii* associated pneumonia and Kaposi's sarcoma; diseases that usually affect only the most severely immunocompromised patients (1). In 1983-84, the cause of AIDS was determined to be a retrovirus, now termed human immunodeficiency virus type-1 (HIV-1) (2,3).

It was later discovered that a similar ailment was affecting patients in western Africa, which lead to the discovery of morphologically similar virus that had a distinct antigenic signature (4). This virus, termed human immunodeficiency virus type-2 (HIV-2), was distantly related to HIV-1 but closely related to a simian virus that induced immunodeficiency in captive macaques (5,6). Upon further investigation, it was found that simian viruses were common in various primate species in sub-Saharan Africa, and within their natural host they are primarily nonpathogenic. Phylogenetic analyses revealed that the simian viruses, collectively called simian immunodeficiency viruses (SIVs), and HIVs derived from a single lineage within the *lentiviridae* genus. Sequencing analyses indicated that the AIDS epidemic was a product of at least two distinct zoonotic transmission events of lentiviruses from primates to humans (7,8). Specifically, it was discovered that the closest viral relative of HIV-1 and HIV-2 were found in chimpanzees and sootey mangabeys, respectively (9,10). The exact nature of the zoonoses is not known, however, the transmission likely occurred when a human was exposed to primate blood/bodily fluids. It has been hypothesized that this type of exposure would be most likely during the hunting of bushmeat (11).

(b) Global Impact

Over 35 years after its discovery, HIV remains a relevant and significant human pathogen with approximately 36.9 million people worldwide that have HIV/AIDs. Despite the huge impact that antiretroviral drugs have had on curbing disease progression and transmission, 22 million infected individuals do not have access to treatment, which includes almost 2 million children. Even in the United States, where the prevalence of HIV has been greatly diminished, there are many barriers to ending the epidemic which include: HIV infected individuals who are unaware of their status, limited health care in populations most at risk, lack of adherence to drug regimens and emergence/spread of drug resistant viruses (12).

(B) Biology of HIV

(a) HIV Replication Cycle- Considering the scope of this thesis, reverse transcription will be the focus of this section.

Structure- HIV is a spherical enveloped virus that contains two single-stranded positive sense RNA genomes, which encode nine open reading frames (ORFs). Three of these ORFs are translated into Gag, Pol, and Env polyproteins. The viral structural proteins are derived from the group-specific antigen (*Gag*) gene, which consists of matrix (MA), capsid (CA), nucleocapsid (NC), and p6; and the envelope (*Env*) gene, which includes gp120 and gp41. The enzymatic proteins of the virus are derived from the polymerase gene (*Pol)* and consist of protease (PR), reverse transcriptase (RT) and integrase (IN). There are six regulatory/accessory proteins which are viral infectivity factor (Vif), viral protein R (Vpr), negative regulatory factor (Nef), trans-activator of transcription (Tat), regulator of expression of viral proteins (Rev), and viral protein u/x (Vpu or Vpx), for HIV-1 and HIV-2, respectively (13). The RNA genomes are enclosed by a conical core made of CA, and tightly bound by NC to prevent damage from host nucleases and detection by pattern recognition proteins (14,15). Also housed within the core are RT, PR, and IN. MA forms a spherical layer between the core and the viral envelope, which is host-derived and decorated with viral Env proteins (13).

Binding & Fusion- Infection begins when the viral Env protein, glycoprotein 120 (gp120), interacts with host surface receptor CD4. Gp120 binding of CD4 induces a conformational change, exposing a site that can bind a second cell surface molecule, CCR5 or CXCR4. Binding of CD4 and a co-receptor allows for the second viral Env protein, gp41, to insert into the cell membrane, and induce fusion of the viral and host cell membrane (16).

Reverse Transcription- Reverse Transcriptase (RT), the viral polymerase of HIV, exists in the mature virion as heterodimer. During the translation of *Gag*, the ribosome will occasionally make a -1 frameshift at the *Gag-Pol* junction resulting in a Gag-Pol polyprotein (17). Proteolytic processing of Gag-Pol by PR yields a 66 kDa subunit (p66), half of which is further processed to yield a 51 kDa subunit (p51). These two proteins form the active RT heterodimer. p66 is the catalytic subunit and contains both the polymerase and the RNase H activity of the enzyme. p51 is catalytically inactive and only serves a structural role. RT has five enzymatic functions which include; 1) RNA-dependent DNA polymerase activity to synthesize the minus (-) strand DNA from the RNA genome, 2) DNA-dependent DNA polymerase activity to synthesize the plus (+) strand DNA, 3) RNase H activity to degrade the RNA genome from the DNA-RNA duplex, 4) strand transfer activity to allow for recombination and template switching and 5) strand displacement activity to complete the synthesis of the linear double stranded DNA (18).

The reverse transcription process (Figure 1), is initiated at the primer binding site (PBS) near the 5' end of the viral genome, where a host tRNA Lys3 acts as primer (19). The RNA-dependent DNA polymerase activity of RT generates the (-) strand strong stop DNA, which consists of the U5 and R region. This 180-nucleotide stretch of DNA-RNA duplex is a substrate for the RNase H activity of RT, which cleaves the RNA of the DNA-RNA, leaving the newly synthesized (-) strand DNA (reviewed in (17)). The next step is strand transfer, where the R region of the (-) strand DNA is transferred to the R region at the 3' end of the viral genome. Synthesis of the (-) strand DNA will continue down the length of the viral genome, with the RNase H activity degrading the RNA template along the way. However, there are two purine rich

sequences, called the central polypurine tract (cPPT) and the polypurine tract (PPT), that are resistant the degradation by RNase H. These regions serve as the primers for the (+) strand synthesis (20,21). Once (+) strand has completed synthesizing 3' of the cPPT, the tRNA primer of the (-) strand is susceptible to RNase H degradation. This allows the second strand transfer to occur between the interaction of the (-) strand and (+) strand PBS (22,23). (+) strand synthesis is completed by the DNA-dependent DNA polymerase activity and strand displacement activity of RT to yield a complete double stranded linear DNA (reviewed in (17)). The process of reverse transcription is highly mutagenic due to the low fidelity of reverse transcriptase, the lack of a RT proofreading mechanism and high rates of retroviral recombination (24-26).

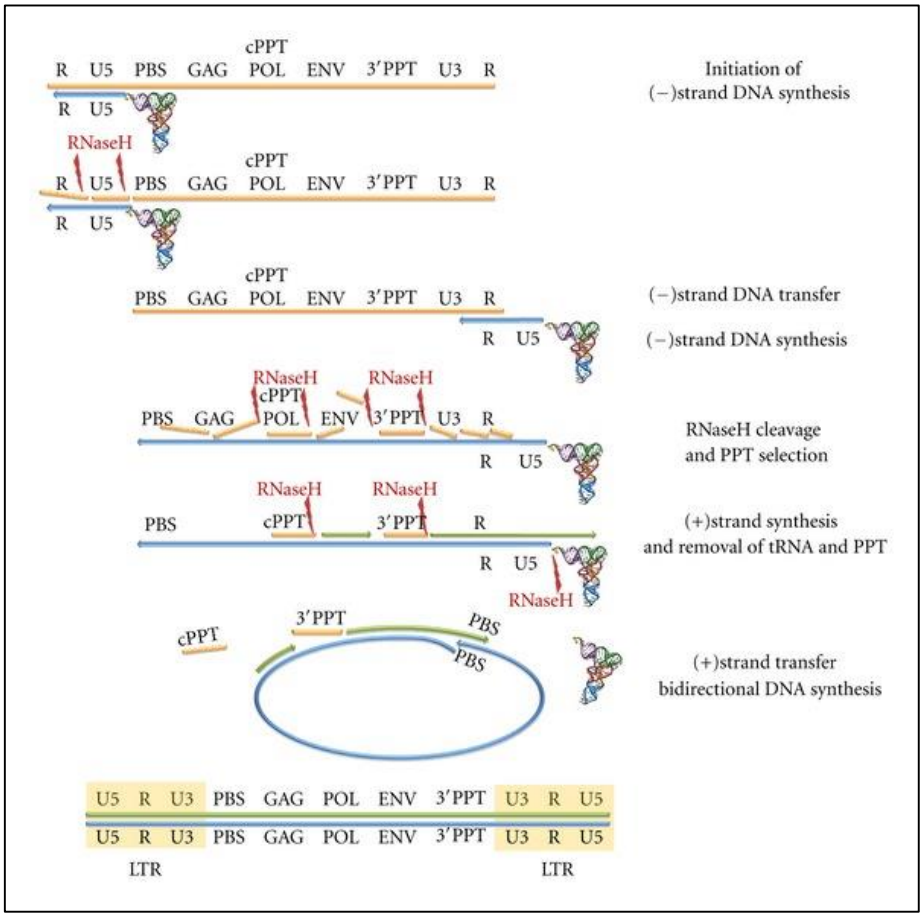

Figure 1: Copyright © 2012 Francesca Esposito et al. Licensed under a Creative Common Attributions 3.0

Figure 1: HIV-1 reverse transcription process. (Copyright © 2012 Francesca Esposito et al. Licensed under a Creative Common Attribution 3.0 (27)) Step 1: host cell tRNALys3 hybridizes to the PBS near the 5′-end of the (+)strand RNA genome (orange). (−)strand DNA (blue) synthesis starts using host tRNALys3 as a primer. DNA synthesis proceeds up to the 5′-end of the RNA genome. Step 2: RNase H hydrolysis of the RNA portion of the RNA:DNA hybrid product exposes the ssDNA product determining the (−)strand strong stop DNA. Step 3: strand transfer of the (−)strand DNA through its hybridization with the R region at the 3′-end of the ssRNA genome and further elongation of the (−)strand DNA. Step 4: DNA synthesis proceeds, and the RNase H function cleaves the RNA strand of the RNA:DNA at numerous points leaving intact two specific sequences (cPPT, 3′PPT) resistant to the RNase H cleavage. Step 5: (−)strand DNA synthesis (green) initiation using PPTs as primers. Step 6: RNase H hydrolysis of the PPT segments and the junction of the tRNA:DNA hybrid, freeing the PBS sequence of the (+)strand DNA. Step 8: strand transfer of the PBS sequence of the (+)strand DNA that anneals to the PBS on the (−)strand DNA. DNA synthesis then continues with strand displacement synthesis. Step 9: the product is a linear dsDNA with long terminal repeats (LTRs) at both ends.

Integration- Upon completion of reverse transcription, the pre-integration complex (PIC) forms, which consists of IN, MA, NC, p6, Vpr and the dsDNA provirus (28). Although the molecular mechanisms are not perfectly understood, these viral factors assist in getting the proviral DNA into the nucleus, a necessity for the infection of nondividing cells (29). Areas of the genome that are decondensed and accessible are targeted for integration (30). IN primes the provirus for integration through 3'-end processing, where two nucleotides are removed from the 3' end of each strand generating a free 3' OH group. Next, IN aligns the host genome and the exposed 3' ends of the viral DNA, allowing the 3' OH to undergo nucleophilic attack of the host DNA phosphodiester bond (31,32).

Replication- RNA polymerase II, numerous host transcription factors and the viral accessory protein Tat initiate efficient transcription of the integrated viral genome (33). Tat and Rev mRNA are exported from the nucleus to the cytoplasm and translated. The Rev protein returns to the nucleus, binds the unspliced mRNAs and assists in their export to the cytoplasm. The full-length RNAs will serve as copies of the viral genome and others will function as mRNAs for the translation of viral proteins (34).

Assembly, Budding & Maturation*:* The Gag polyprotein is the mediator of virion assembly through its interactions with the RNA genome, binding of the plasma membrane and protein-protein interactions necessary for viral morphology. After all of the essential viral components are assembled at the plasma membrane, Gag recruits host ESCRT factors which are required for budding and membrane fission. Immediately after the virus buds from the cell, virion maturation is driven by proteolytic processing of the polyproteins by PR, which yields all of the components of an infectious virion (reviewed in (35)).

(b) HIV pathogenesis

Transmission & Acute Infection- HIV transmission can occur if bodily fluids, such as blood, semen, pre-seminal/rectal/vaginal fluid or breast milk, of an HIV infected individual

comes in contact with another individual's mucous membranes, damaged tissue or blood. The transmission of the virus can occur through oral, vaginal and anal sex, sharing of needle/syringes, mother to child and blood transfusions (36). A single founder virus establishes infection and the founder is unique from the viral quasispecies in that its co-receptor preference is CCR5 rather than CXCR4 (37). Three to nine weeks after infection, the viral load peaks, CD4 cells will drop, and patients may experience flu-like symptoms. Host immunity eventually starts to control the virus and viremia reaches a steady state. At this stage, which is also referred to as clinical latency, the virus is actively replicating at the rate of 1 billion virions per day, but the host immunity is capable of replenishing the immune cells that are killed and suppressing the infection (38,39).

Viral Tropism- As mentioned above, the founder virus that initiates infection is often CCR5-tropic. CCR5 is expressed on macrophages, dendritic cells, and memory T cells, and therefore CCR5-tropic viruses are often referred to as macrophage-tropic (M-tropic). The primary usage of this co-receptor during the initial infection is evident by the high resistance of individuals who are homozygous defective for the CCR5 gene to HIV infection (40). Replication of the founder virus leads to diversification of the quasispecies, and in most cases a seroconversion, from CCR5 usage to CXCR4 (T-cell-tropic), will eventually occur (16). Naïve CD4+ T cells robustly express CD4 on their surface so this seroconversion, which leads their infection and subsequent depletion, is often associated with clinical decline (41).

Cellular Permissivity- Lentiviruses, such as HIV and SIV, are unique from the *Retroviridae* family in that they can infect both nondividing and dividing cells, whereas other retroviruses, such as oncoretroviruses, only infect the latter (42). Nondividing cells, such as macrophages and resting CD4 T cells, are far less permissive to HIV infection than actively dividing cells, such as activated CD4 T cells (43,44). This restriction is owed to cellular differences between dividing and nondividing cells, which include cell cycle status, 2’ deoxynucleotide (dNTP) levels, and expression of specific cellular proteins (45-47). dNTP levels, which have strong implications for this dissertation, are maintained at very low levels in

macrophages and resting CD4 T cells due to the high expression of Sterile alpha motif and histidine-aspartic domain containing protein 1 (SAMHD1) and low ribonucleotide reductase (RNR) activity (which will be discussed in detail in Chapter I, Part C) (47,48). Viral restriction in these cells can be overcome by increasing cellular dNTP levels, through degradation of SAMHD1 or addition of exogenous deoxynucleosides, which indicates the permissivity of nondividing cells is controlled by their low dNTP concentrations (49).

The importance if macrophages in HIV pathogenesis is often overlooked, as CD4+ T cells are the majority of infected cells and their decline leads to AIDS. From a clinical standpoint, the infection of macrophages is important due to the increased prevalence of HIV-1 associated neurocognitive disorder (HAND). This disorder causes cognitive impairment, which stems from chronic inflammation of the brain due to infection of brain macrophages, known as microglia (50-52). Importantly, HIV has evolved to prolong the survival of infected macrophages, which can survive weeks and even years after infection. On the other hand, CD4+ succumb relatively quickly, within 1-2 days, due to their sensitivity to the cytopathic effects of the virus (38,53,54). These long-lived infected macrophages may also serve as a viral reservoir (54,55).

Progression to AIDS- Although HIV can remain clinically latent for years, the immune system eventually exhausts its ability to replenish CD4 T cells and patients become vulnerable to opportunistic infections (reviewed in (56)). Two benchmarks clinically define whether a patient has progressed to AIDS: 1) CD4 cell count below 200 cells per cubic millimeter of blood or 2) one or more opportunistic illnesses regardless of CD4 count (57).

Interestingly, HIV-2 has a notably longer asymptomatic phase and slower progression to AIDS than HIV-1 (58). The reduced pathogenicity of HIV-2 is not completely understood. One study has indicated that HIV-1 and HIV-2 infected individual have similar amounts of integrated viral DNA however viral loads are substantially higher in HIV-1 patients (59). The attenuation of

pathogenesis in HIV-2 is of clinical interest because it is a potential exploit in the viral replication of those infected with HIV-1.

(c) Highly Active Antiretroviral Therapy (HAART)

The first drug to be developed for HIV, azidothymidine (AZT), was a nucleoside analog that induces chain termination. Although AZT effectively treated patients with the virus, treatment with a single antiretroviral quickly led to resistance and demonstrated that HIV was highly mutable and able to quickly escape drug pressure (60,61). In 1996, two labs published data indicating combination treatment, with two nucleoside reverse transcriptase inhibitors (NRTIs) and a protease inhibitor (PI), greatly improved treatment outcomes (62,63). Clinicians quickly adopted this three drug regimen and AIDS related hospitalizations and death dramatically declined 60-80% (64).

The current standard of care for HIV infected individuals is to immediately begin, despite viral load or CD4+ T cell count, a regimen of highly active antiretroviral therapy (HAART). HAART treatment suppresses viral loads to nearly undetectable levels, which means that treatment is also very good at preventing disease transmission. The most common HAART regimen for a treatment-naive patient consists of two NRTIs plus a third active antiretroviral (ARV) drug from one of three drug classes: an integrase strand transfer inhibitor (INSTI), a non-nucleoside reverse transcriptase inhibitor (NNRTI), or a PI (65).

Patient adherence to their antiretroviral drug regimen is of utmost importance for prevention of resistance and transmission. However, oral treatment for chronic diseases have very poor adherence rates, with about 50-70% nonadherence (66). Studies of long-acting ARVs (injectables and subcutaneous implants) are being conducted to determine the safety and efficacy of these alternatives which could vastly improve adherence (reviewed (67)).

NRTIs- The HIV viral polymerase, RT, is a primary target for HIV treatment. Approved HAART regimens consist of two classes of RT inhibitors; NRTIs and NNRTIS. Both classes prevent the enzymatic activity of RT albeit by different mechanisms. The focus of this thesis will

be NRTIs, which compete with the natural substrate, dNTPs, for binding of the HIV RT active site. Generally, NRTIs are given in their nucleoside form, which is not active, so they can effectively be transported into the cell. Once in the cell, they compete with endogenous nucleosides for phosphorylation by cellular kinases to yield the active triphosphate form (NRTI-TP). The low fidelity of RT does not discriminate dNTPs from dNTPs and these molecules are effectively incorporated into viral DNA. Importantly, high fidelity host polymerase can exclude and proofread NRTI-TPs making incorporation into host DNA less likely. NRTI-TPs are obligate chain terminators because they lack a 3' hydroxyl on their sugar moiety, which prevents the formation of a phosphodiester bond with the next incoming dNTP and inhibits extension of the viral DNA (27,68). Currently there are seven NRTIs that are FDA approved and clinician recommended including abacavir (ABC), didanosine (ddI), emtricitabine (FTC), lamivudine (3TC), stavudine (d4T), tenofovir disproxil fumarate (TDF), and zidovudine (AZT) (69).

All of the FDA approved NRTIs act as obligate chain terminators, however NRTIs with other mechanisms have been discovered. Delayed chain terminators (such as clofarabine-TP, the focus of Chapter II) have a 3' OH group and therefore additional nucleotides can be incorporated. However, the noncanonical structure of the NRTI eventually creates enough perturbation of the elongating primer that it loses the ability to either bind the next nucleotide or create the next phosphodiester bond (70-72). Importantly, the incorporation of nucleotides to after these NRTIs prevents 3' end nucleotide excision by HIV RT (71,73).

Lethal mutagens (such as 5-azacytidine and decitabine, the focus of Chapter III) are another class of NRTIs that have not been actively pursued in the clinic. These NRTIs allow for the further incorporation of dNTPs, but they induce a significant increase in nucleotide mismatch. Fidelity and mutagenesis maintain a necessary balance so that viable progeny are created and adaptation to host immunity and drugs is possible (74,75). HIV-1, like many other viruses, replicates very close to its error threshold which gives the virus a great adaptive advantage. However, this also means that even a small increase in mutagenesis can lead to an inviable

population (lethal mutagenesis). This equilibrium between mutation and fidelity is very important for antivirals that aim to induce lethal mutagenesis of viral populations (74,76,77).

Viral Mutagenesis & NRTI Resistance- The low fidelity of RT, high recombination rates, and exorbitant virion production all play a role in viral mutagenesis. The high rates of viral mutagenesis produce an extraordinary amount of noninfectious virions, however it also allows the viral quasispecies to sample sequences that are capable of evading host immunity and creating drug resistance (26).

The two mechanisms of NRTI resistance are excision and exclusion. Mutations utilizing the excision mechanism were the first to be discovered in response to AZT treatment and they are often referred to as thymidine analog mutations (TAMs). TAMs occur in two distinct sets of combined mutations; 1) M41L, L210W, T215Y and sometimes D67N or 2) D67N, K70R, T215F, and K219E/Q (78-81). These mutations increase the ability of RT to remove AZT from the terminated end of the viral DNA. This may seem counterintuitive because RT lacks a 3'-5' proofreading capability, however this reaction is instead a 'reverse polymerization reaction' using ATP as pyrophosphate donor (82,83).

The other mechanism of NRTI resistance is the exclusion mechanism, which increases the capability of RT to discriminate the canonical substrates, dNTPs, from NRTI-TPs. These mutations have been described for multiple NRTIs and usually involve Q151M and a complex of other mutations including A62V, V75I, F77L, and F116Y (84). However, the distinct structure of each NRTI is capable of imparting specific 'signature' mutations that are precise for the exclusion of each NRTI-TP. For example, 3TC and FTC resistance is often associated with M184V/I, where the V/I at residue 184 results in steric hindrance with the NRTI-TP but not dCTP (85). Interestingly, the low fidelity of RT that assists in its rapid evolution is often compromised with these mutations, and RT increases in selectivity for dNTPs and overall fidelity (86).

Lifelong treatment and poor adherence are two factors in the emergence of resistance. Transmission of drug resistant viruses is also a serious concern, with about 50% of viremic patients harboring M184V virus and 6-16% of patients being infected with a virus that is resistant to at least one drug (87,88). Therefore, novel NRTIs such as delayed chain terminators and lethal mutagens, which are often effective against drug resistant viruses, are attractive clinical pursuits.

(d) Viral Reservoirs & HIV Cure

HAART has greatly reduced fatality and transmission of HIV, however upon treatment cessation or development of resistance, the virus can quickly rebound due to a reservoir of cells that maintain an integrated copy of HIV (89,90). This viral persistence has led to the search for an HIV cure, which would require the clearance or permanent suppression of all latently infected cells.

The only person to have been successfully cured of HIV-1 is Timothy Brown, who is better known as the Berlin patient. Brown was HIV positive and also had acute myeloid leukemia. To treat the cancer, he underwent total body irradiation followed by a bone marrow transplant. Doctors found a donor who had a rare homozygous mutation in the HIV co-receptor CCR5 that imparts high resistance to HIV infection. The complete loss of Brown's bone marrow cells in conjunction with transplant cells that were resistant to infection, the Berlin Patient remains HIV free to this day (91,92). This case shows that a cure is possible, however, not attainable for almost all other HIV-infected individuals. Following the success of the Berlin patient, researchers tried to cure two men now known as the 'Boston Patients'. Similar to Timothy Brown, these patients had both HIV and cancer. Both patients were heterozygous for the CCR5 mutation that contributes to HIV resistance but received transplants from donors that were wild-type for CCR5. Although, HIV RNA and DNA remained undetectable until 12 and 32 weeks after HAART cessation, both patients experienced viral rebound (93,94).

The Boston patients, along with other unsuccessful clinical trials, have made it very clear that a cure is not attainable without addressing the viral reservoir (94,95). Although quite

controversial, the viral reservoir is believed to consist of primarily long-lived memory CD4+ T cells, but also may include naïve CD4+ cells, T follicular helper cells, and macrophages, which exist in an array of tissues including the secondary lymph nodes, spleen, gut mucosa, urogenital tract and brain. It is widely accepted that memory CD4+ T cells are the primary reservoir, but the contribution of each of these cell types and tissues to the persistence of replication competent virus is still under intense investigation (96,97). Understanding which cells types contain replication competent virus and which tissues they reside will be necessary to advance cure research.

(C) Nucleic Acid Metabolism

(a) Nucleotide Biosynthesis

Nucleotides are crucial to many essential life processes such as the replication of genomic DNA, transcription of mRNA for protein synthesis, certain signal transduction pathways, mitochondrial function and more. Cellular nucleotide biosynthesis has two pathways, the *de novo* pathway and the salvage pathway. *De novo* means 'from scratch' and this pathway is comprised of a series of elementary reactions that are repeated with variations to yield different nucleotides. On the other hand, the salvage reaction consists of recovering preformed bases and reconnecting them to a ribose moiety. Both the *de novo* and salvage pathways synthesize ribonucleotides which are then used as the building blocks to create 2' deoxyribonucleotides (dNTPs) (98).

(b) Deoxyribonucleotide Metabolism

Cellular dNTP regulation is necessary to maintain a balanced pool of dNTPs, which ensures fidelity during genomic replication. Even modest disruptions to the ratio of dNTPs can lead to poor DNA polymerase fidelity and proofreading, which in turn can result in genome instability and mutagenesis (99-101). The maintenance of the correct dNTP concentrations is a complex process involving many proteins. Two major players are the enzymes: ribonucleotide reductase (RNR) and sterile alpha motif and histidine-aspartic domain containing protein 1

(SAMHD1). RNR is responsible for the *de novo* synthesis of dNTPs from ribonucleotides, whereas SAMHD1 is responsible for the hydrolysis of dNTPs to dNs and triphosphates. These two enzymes, working in concert, maintain cellular dNTP concentrations at the proper levels for the genomic replication and DNA repair necessary for every cell (102).

(c) Ribonucleotide Reductase

Function- RNR is the rate-limiting enzyme in *de novo* dNTP synthesis and catalyzes the reduction of the 2' hydroxyl of ribonucleoside diphosphates (NDPs) to generate deoxyribonucleoside diphosphates (dNDPs). Subsequently, dNDPs are phosphorylated by cellular kinases to their triphosphate form, deoxynucleoside triphosphates (dNTPs), which can then be used for cellular DNA replication, mitochondrial DNA replication, and DNA repair (98).

Structure- Human RNR consists of two subunits, α and β to form the active holoenzyme. The *Rrm1* gene produces the α, or R1 subunit, which harbors the catalytic active site and two distinct allosteric sites. The *Rrm2* and *p53R2* genes produce two distinct isoforms of the β subunit, known as R2 and p53R2, respectively. Both β subunits contain a di-iron cofactor and protein tyrosyl radical that is transferred to the α subunit during catalysis. Allosteric effectors induce the dimerization of R1, which then forms a tetramer consisting of two R2 or two p53R2 subunits ($\alpha_2\beta_2$) (103).

Expression & Regulation- The expression and regulation of RNR is tightly controlled by cell cycle status and nucleotide levels, respectively (46). Prior to S phase, when the cell is preparing to synthesize another copy of its genome, RNR activity increases to ensure there are sufficient dNTPs for replication. The transcription of the R1 and R2 subunit is cell-cycle dependent, with undetectable mRNA levels during G_0/G_1 and the highest levels of mRNA during S phase (104). The half-life of R1 is greater than 20 hours and therefore R1 remains at a consistent level in all cells, and in excess in proliferating cells (105). Expression of R2 is upregulated during S phase, and repressed during G_1 by two distinct promoter regions (106,107). Additionally, R2 has a shorter half-life (3 hours) which is induced by proteolysis after a cell

enters mitosis (108). The stricter regulation and shorter half-life of R2 limits the enzymatic activity of RNR. The p53R2 subunit, the alternative β subunit to R2, is expressed constitutively at low levels and is not affected by cell cycle. Follwoing DNA damage, cells undergo G_1 arrest and R2 expression is repressed and the protein undergoes proteolysis. This arrest induces the transcriptional activation of *p53R2* allowing for the synthesis of dNTPs necessary for DNA damage repair (109,110).

As shown in Figure 2, the allosteric regulation of RNR is extremely sophisticated with four distinct allosteric effectors and two allosteric sites, both of which are contained in the R1 subunit. The specificity site (s-site), shown as allo-site 2 in Figure 2, can bind ATP, dATP, dTTP, and dGTP, and binding of each individual effectors induces a unique conformational change causing the catalytic site more likely to bind one substrate over another (103,111). The s-site does not efficiently bind dCTP and the regulation of dCTP and dTTP is controlled by dCMP deaminase. This extensive regulation ensures that the dNTP pool is balanced, and no dNTP is produced in excess, protecting the fidelity of DNA replication (111,112).

The activity site (a-site), shown as allo-site 1 in Figure 2, acts as a master switch capable of turning the enzyme off if dNTP concentrations become too high (103). The a-site is regulated by ATP, which is an activator, and dATP, which is an inhibitor. ATP has similar affinities for both the s-site and the a-site. On the other hand, dATP has 10-20 times lower affinity for the a-site than the s-site, making it capable of acting as a s-site regulator when it is at low concentrations. At higher concentrations, dATP will shut down enzyme activity (113-115). The mechanism behind the a-site control of enzyme activity is oligomerization. If dATP levels are high, and the a-site is bound, then the R1 subunit will hexamerize and form a complex that is incapable of reducing rNDPs (116).

(d) SAMHD1

The cellular significance of SAMHD1 was first realized when it was found to be one of seven genes associated with Aicardi-Goutières syndrome (AGS), a genetically defined type I

interferonpathy (117). Two years later, SAMHD1 became of great interest to the HIV field after it was discovered to be an HIV restriction factor and a dNTPase (118-120). In this section, the focus will be on the most well characterized function of SAMHD1, its dNTPase activity.

Function*:* The exact cellular role of Sterile alpha motif and histidine-aspartic domain containing protein 1 (SAMHD1) has yet to be fully realized, however current data is definitive in that SAMHD1 is the only known eukaryotic deoxynucleoside triphosphate triphosphohydrolase (dNTPase), meaning it converts dNTPs to dNs and triphosphates. Additionally, it has been implicated in cell cycle, apoptosis, innate immune sensing, RNA binding/nuclease activity and endogenous retrotransposon inhibition; albeit the role of SAMHD1 in these processes is much less defined (121-125).

Structure: SAMHD1 has three defined regions; the N-terminus (residues 1-109), the catalytic core (residues 110-599), and the C-terminus (residues 600-626). The N-terminus consists of the sterile alpha motif (SAM) domain. SAM domains are historically involved in protein-protein and protein-nucleic acid interactions; however, the exact biological function of the SAM domain of SAMHD1 has yet to be elucidated. This region is known to have undergone positive evolutionary selection, which is a consequence of its interaction with some lineages of SIV Vpr and SIV/HIV-2 Vpx (126,127).

The catalytic core of SAMHD1 harbors the dNTPase active site as well as two allosteric sites. In physiological conditions, allosteric site 1 will always be bound to GTP or dGTP causing SAMHD1 to dimerize. The second allosteric site can bind any dNTP and induces the formation of a long-lived catalytically active tetramer (128-130). The active site consists of two sets of histidine and aspartate residues (His206, Asp207 and His167, Asp 311) which coordinate a metal cation necessary for catalysis. The active site can bind any of the four dNTPs, albeit with varying affinities (128,131).

The C-terminus of SAMHD is critical for stabilization of the SAMHD1 tetramer and also contains a binding site for the cyclinA2-CDK complex, which is necessary for phosho-regulation

of the protein (132,133). Similar to the N-terminus, the C-terminus has undergone positive selection due to interaction with Vpr/Vpx, indicating that viral proteins and host SAMHD1 have been coevolving for some time (126,127).

***Expression and Regulation**:* SAMHD1 is constitutively expressed at basal levels in all nucleated hematopoietic cells; however, SAMHD1 expression is especially robust in monocytes, monocyte derived macrophages, monocyte derived dendritic cells (134). The most predictable regulator of SAMHD1 expression is the cell cycle; with peak expression levels in quiescent cells, and diminished expression in cycling cells (102).

Similar to RNR, the regulation of SAMHD1 is complex and involves allosteric activation by nucleotides. As shown in Figure 2, the active form of SAMHD1 exists as a tetramer, which requires the binding of nucleotides at two distinct allosteric sites. The first allosteric site (allo-site 1), is specific for a guanine base and triphosphate but can accommodate both dGTP and GTP. The second allosteric site, allo-site2, requires a dNTP but can accommodate any of the four bases, with the highest affinity for dATP (128). The prevalence of GTP in all cells indicates that dNTP concentrations and binding of A2 site control the activation of the protein (135). Unlike RNR, there is no effect of secondary allosteric site binding on the affinity of each nucleotide for the active site. The degradation of nucleotides is determined by affinity of each nucleotide for the tetramer active site, with dATP having the lowest affinity for the active site (128).

SAMHD1 is also regulated via phosphorylation at residue T592, by the complex of cyclinA2 and cyclin-dependent kinase 1 (cyclinA2-CDK1) (132,136). Cyclin-CDK complexes are master controllers of cell cycle, and it was further discovered that other cyclin-CDK complexes such as cyclinE-CDK2 and cyclinD3-CDK6 also function in SAMHD1 phosphorylation (137). The role of SAMHD1 phosphorylation has been contentious due to the use of various cell models, many of which are transformed and have a dysfunctional cell cycle. However, the general consensus from these cell models is that phosphorylation does not affect dNTP degradation but does have an effect on retroviral restriction (123,136,138,139). Structural

modeling indicates T592 phosphorylation leads to an instable tetramer, which will dissociate into the catalytically inactive dimer/monomer faster than the unphosphorylated protein. Further biochemical analyses confirmed this result, indicating that phosphorylated tetramer is less stable and more sensitive to dNTP fluctuations (140,141).

SAMHD1 is also regulated by retroviral infection. The degradation of SAMHD1 is induced by HIV-2/SIV Vpx, or in the case of some SIVs, Vpr (126,142). Once a host cell is infected, Vpx associates with numerous host factors involved in proteasomal degradation (143). Vpx binds SAMHD1 and depending on the viral species this interaction can occur at the N-terminus or C-terminus (126,127) . Interaction with Vpx and host factors results in the proteasomal degradation of SAMHD1, inducing an increase in cellular dNTPs. The loss of SAMHD1 and subsequent increase in cellular dNTP levels increases the permissiveness of nondividing cells (49). Interestingly, SAMHD1 is not counteracted by HIV-1, which lacks Vpx, although providing Vpx in *trans* increases the infection of myeloid cells (118,119). The nonpermissiveness of myeloid cells may have been instrumental in HIV-1 immune escape strategy and potentially be related to the difference in pathogenesis between HIV-1 and HIV-2.

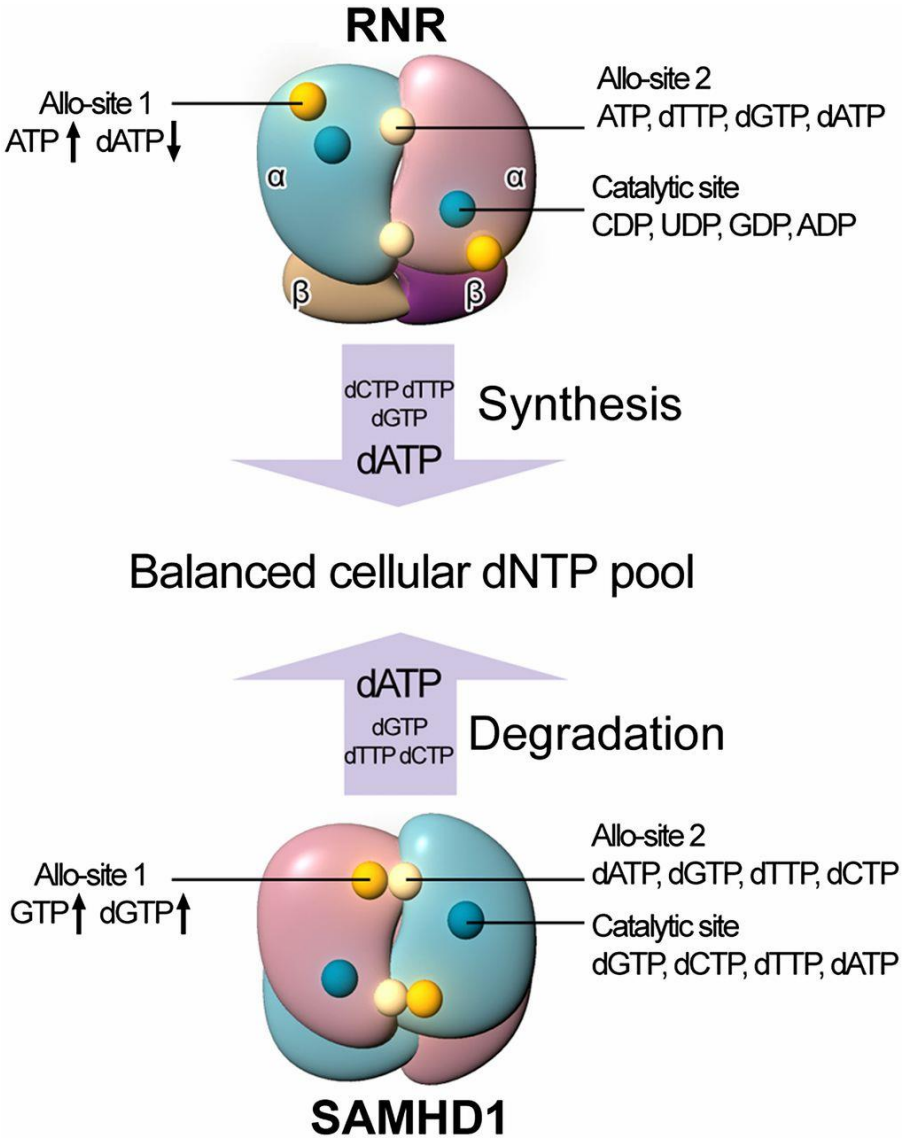

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Figure 1.2 SAMHD1 and RNR regulate cellular dNTP pool together. RNR and SAMHD1 are responsible for the production and degradation of dNTPs, respectively. Both of them have three specific nucleotides binding sites as indicated, with the types of the nucleotides that can occupy the sites marked on the side. The concentrations of cellular dNTPs are sensed and regulated by both of the enzymes. dATP is produced last by RNR and degraded last by SAMHD1 (128).

(e) Nucleotide Regulation and Disease

The stringent regulation of RNR and SAMHD1 indicate that dNTP concentrations need to be maintained at the proper concentrations at the appropriate time for cellular function. At the organismal level, dNTP dysregulation is linked with the progression of cancer and increased susceptibility to viral infections.

Cancer- At the cellular level, imbalanced dNTPs can lead to enhanced mutagenesis and cell cycle dysregulation which in turn lead to a cancerous phenotype. Impairment of dNTP homeostasis is a common biomarker for cancerous cells and not surprisingly mutations in RNR (most commonly R2) and SAMHD1 have been found in a variety of cancer types (144,145). The molecular mechanisms of SAMHD1 and implications for cancer will be discussed in Chapter V. Due to its essential role in cancer development and progression, targeting of nucleotide metabolism and nucleoside analogs have been the backbone of cancer therapy for decades (146). Two anticancer drugs that effect or/utilize RNR, clofarabine and 5-azacytidine, will be discussed in Chapter II & III, respectively.

Viral Infections- Viral pathogens that require dNTPs to replicate their genome have evolved various mechanisms to ensure substrate availability for replication. As previously discussed, HIV-2/SIV overcome low dNTP environment of nondividing cells by inducing the degradation of SAMHD1 with Vpx, which increases cellular dNTPs (118,119). Large dsDNA viruses, such as vaccinia virus and herpes simplex virus, encode dNTP biosynthesis proteins such as a viral RNR and thymidine kinase to ensure sufficient nucleotides for viral replication (146,147). Hepatitis B virus is quite sophisticated in its dNTP regulation through upregualtion of host RNR, while also limiting the host consumption of dNTPs by attenuating DNA damage repair (148,149). Importantly, viral alterations to the host cell cycle and dNTP regulation can lead to cancerogenesis (150). Similar to cancers, the viral dependence on nucleotides provides an exploitable clinical target, and nucleoside analogs, as well as agents that inhibit viral nucleotide biosynthesis, have been a cornerstone of treatment (151).

CHAPTER II: Dual anti-HIV mechanism of clofarabine

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M.Daly contributed the data for Figure 1, Figure 2 (parts A, B,C), Figure 3 (A,C,D), Figure 4, Supplemental Figure S1 and co-wrote the manuscript.

Abstract

BACKGROUND: HIV-1 replication kinetics inherently depends on the availability of cellular dNTPs for viral DNA synthesis. In activated CD4(+) T cells and other rapidly dividing cells, the concentrations of dNTPs are high and HIV-1 reverse transcription occurs in an efficient manner. In contrast, nondividing cells such as macrophages have lower dNTP pools, which restricts efficient reverse transcription. Clofarabine is an FDA approved ribonucleotide reductase inhibitor, which has shown potent antiretroviral activity in transformed cell lines. Here, we explore the potency, toxicity and mechanism of action of clofarabine in the human primary HIV-1 target cells: activated CD4(+) T cells and macrophages.

RESULTS: Clofarabine is a potent HIV-1 inhibitor in both activated CD4(+) T cells and macrophages. Due to its minimal toxicity in macrophages, clofarabine displays a selectivity index over 300 in this nondividing cell type. The anti-HIV-1 activity of clofarabine correlated with a significant decrease in both cellular dNTP levels and viral DNA synthesis. Additionally, we observed that clofarabine triphosphate was directly incorporated into DNA by HIV-1 reverse transcriptase and blocked processive DNA synthesis, particularly at the low dNTP levels found in macrophages.

CONCLUSIONS: Taken together, these data provide strong mechanistic evidence that clofarabine is a dual action inhibitor of HIV-1 replication that both limits dNTP substrates for viral DNA synthesis and directly inhibits the DNA polymerase activity of HIV-1 reverse transcriptase.

KEYWORDS: Clofarabine; Human immunodeficiency virus (HIV); Nucleoside/nucleotide analogue; Reverse transcription; Ribonucleotide reductase

Background

Deoxyribonucleoside triphosphates (dNTPs) are essential for the genomic DNA replication of all organisms. In mammalian cells, two pathways supply the cell with dNTPs: the de novo synthesis pathway and the salvage pathway. Ribonucleotide reductase (RNR) is the rate-limiting enzyme in de novo dNTP synthesis and acts by reducing ribonucleotides to deoxyribonucleotides. Nucleotide levels and cell cycle status tightly regulate the expression and activity of RNR [1, 2]. Prior to S phase, RNR activity greatly increases ensuring a sufficient supply of dNTPs for DNA replication. However, cells that are in nondividing or resting states display restricted RNR activity, which results in a low dNTP environment [3, 4]. In contrast, transformed/cancer cells, which are rapidly dividing with uncontrolled cell cycles, have significantly higher dNTP levels compared to normal dividing cells, and elevated dNTP levels are considered a biochemical marker for cancer cells [4, 5].

Various intracellular pathogens that synthesize DNA, including human immunodeficiency virus type 1 (HIV-1), use cellular dNTPs for their genome replication. Two primary target cells of HIV-1 are activated CD4+ T cells and macrophages. Activated CD4+ T cells are dividing cells that contain abundant dNTPs (1–5 μM), synthesized by the highly expressed RNR, to support the replication of their genome [6–8]. On the other hand, macrophages are terminally differentiated and nondividing. Due in part to their extremely low RNR expression they have substantially lower dNTPs (20–50 nM) than dividing cells [7–9]. HIV-1 replication kinetics is slower in macrophages compared to activated CD4+ T cells [8, 10]. We previously reported that the low dNTP pools found in macrophages kinetically delays HIV-1 reverse transcription, suggesting that limited dNTPs serve as a restriction mechanism against HIV-1 in nondividing cells [8].

Recently, SAM domain and HD domain containing protein 1 (SAMHD1) was identified as a potent myeloid-specific host restriction factor of HIV-1 that depletes cellular dNTPs [11],

which subsequently suppresses HIV-1 replication in nondividing cell types such as macrophages [12–14]. Interestingly, unlike HIV-1, HIV-2 and many SIVs encode a viral accessory protein, viral protein X (Vpx), that counteracts SAMHD1 and promotes retroviral replication in macrophages by elevating cellular dNTPs [13, 15, 16]. In addition to SAMHD1, other dNTP pool modulators have been shown to effect HIV reverse transcription. Most notably, cyclin-dependent kinase p21 inhibits HIV replication by repressing the expression of an alternative RNR subunit called RNR2 [17]. Overall, these data indicate that sufficient dNTP levels are necessary for viral reverse transcription and depleting dNTPs restricts viral replication.

Clofarabine is an FDA approved RNR inhibitor (RNRI) used in the treatment of acute lymphoblastic leukemia. As a purine nucleoside analog (Fig. 1a), clofarabine is transported into cells by nucleoside transporters and is phosphorylated by host enzymes to the active forms of the drug, clofarabine di- and triphosphate [18]. Both clofarabine di- and triphosphate inhibit RNR by binding the catalytic and the allosteric regulatory sites inducing hexamerization of the large subunit of RNR and subsequently preventing formation of the active enzyme. Due to the necessity of RNR in de novo dNTP synthesis, this inhibition causes a reduction in endogenous dNTPs, which in turn can inhibit DNA synthesis due to limited substrate availability [19, 20]. In addition to RNR inhibition, clofarabine triphosphate (clofarabine-TP) can also be incorporated as an adenosine analog by DNA polymerase-α and -ε and can induce chain termination [21, 22]. Other DNA polymerases can also incorporate clofarabine albeit at a much-reduced rate that is not of biological significance. The mechanism for chain termination of clofarabine is not entirely clear; as clofarabine has a 3′ OH, it is not an obligate chain terminator like azidothymidine (AZT). It has been observed that incorporation of clofarabine-TP reduces the rate at which the next nucleotide will be incorporated and incorporation of two consecutive clofarabine-TPs makes it extremely unlikely that the DNA chain will be further elongated. One current hypothesis for this inhibition of extension is that the 2′ fluorine atom may affect the reactivity of the 3′ OH

and/or the quaternary structure of the DNA such that the polymerase fails to efficiently make the next bond [22].

We have previously demonstrated that clofarabine has anti-HIV activity in cell culture using a transformed cells line [23], however these studies did not examine the mechanism of action, or the anti-HIV activity and toxicity in the primary target cells of HIV: activated CD4+ T cells and macrophages. Our biochemical and cell culture data indicate that clofarabine effectively blocks HIV-1 replication by two distinct mechanisms: (1) reduction of cellular dNTPs and (2) direct incorporation by and inhibition of HIV-1 reverse transcriptase, with very limited toxicity in macrophages.

Results and discussion

Anti-HIV-1 activity and cytotoxicity of clofarabine in human primary target cells

Clofarabine, a purine nucleoside analog (Fig. 1a) and RNRI, is an FDA approved anticancer compound that we have recently shown to have antiretroviral potency in a transformed cell line [23]. Here, we examined the anti-HIV-1 activity of clofarabine in the primary human cells that are targets of HIV-1: activated CD4+ T cells and monocyte derived macrophages (MDMs). Cells were isolated from five healthy donors, pretreated with varying concentrations of clofarabine for 8 h, and then infected with HIV-1 pseudotyped with vesicular stomatitis virus G protein (VSV-G). The construct used expresses the full-length HIV-1 genome, with a frameshift in *env* and two fluorescent protein genes, *mCherry* and *enhanced GFP* (*EGFP*), replacing a portion of *rev* and *nef* [24]. Cells were analyzed with flow cytometry at 5 days (MDMs) or 3 days (T cells) after the addition of virus, and infected cells were determined by EGFP expression. Macrophages, as expected, showed a more restricted HIV-1 infection than the CD4+ T cells; however, however similar infectivity was achieved by using five times the amount of virus in MDMs (Additional

file 1: Figure S1A). As shown in Figs. 1b and c (blue lines), clofarabine caused a concentration-dependent decrease in HIV-1 infection in both cells types, with half maximal inhibitory concentration (IC50) values of 21.6 nM [95 % confidence interval (95 % CI) 17.4–25.8 nM] in macrophages and 60.3 nM (95 % CI 24.1–96.5 nM) in activated CD4+ T cells. This three-fold increase in potency in macrophages compared to T cells is surprisingly minor—in the low dNTP environment of macrophages, we expected that the ratio of clofarabine-DP and -TP to dADP and dATP, respectively, would be much higher than that found in T cells, and therefore considerably more potent. However, this analysis is complicated by the fact clofarabine-TP has recently been identified as a substrate for SAMHD1, which is highly expressed in macrophages but not T cells [25].

We also determined the cytotoxicity of clofarabine in activated CD4+ T cells and macrophages (red lines in Fig. 1b, c) using the XTT assay, and found that macrophages are far more resistant to clofarabine-induced toxicity than activated CD4+ T cells, with CC50 values of 6.8 μM (95 % CI 3.2–9.4 μM) and 854 nM (95 % CI 713–996 nM), respectively. Additional toxicity assays, including analysis of membrane integrity and cell size, were performed and supported this result (Additional file 1: Figure S1B–E).

This eight-fold difference in cytotoxicity indicates that macrophages are significantly more resistant to the toxic effects of clofarabine. The difference in clofarabine toxicity in macrophages and T cells may be due to multiple factors. One possibility is that T cells are actively dividing which provides an opportunity for clofarabine-TP to be incorporated into their genome [26]. In cancer cells this genomic incorporation of clofarabine-TP has been show to be toxic. Additionally, nucleotide starvation due to RNR inhibition and DNA damage response can induce cell cycle arrest and potentially lead to apoptosis [27–29]. These factors would not necessarily affect macrophages because they are nondividing state and therefore not replicating their genome and macrophage nucleotide levels are already extremely low compared to dividing

cells. Another possible explanation is that clofarabine-TP, along with other dATP analogs, is known to induce mitochondrial toxicity by altering the mitochondrial transmembrane potential [30]. SAMHD1, which is highly expressed in macrophage but not T cells, may be degrading clofarabine-TP and therefore limiting the effect of mitochondrial toxicity in MDMs.

Despite the fact that clofarabine-TP can be degraded by SAMHD1, clofarabine remains very potent in macrophages (IC50 = 20.3 nM) and has limited cytotoxicity in this cell type. The selectivity index (SI, CC50/IC50) for clofarabine in macrophages is 314.8, 22-fold greater than the SI in activated CD4+ T cells (Fig. 1d), suggesting that clofarabine is a highly selective inhibitor of HIV-1 specifically in macrophages.

Effect of clofarabine on cellular dNTP levels and HIV-1 DNA synthesis

We previously reported that the dNTP concentration in activated CD4+ T cells (1–5 μM) is above the Km value of HIV-1 RT (100–200 nM) [8, 31]. On the other hand, macrophages have low dNTPs (50 nM) with concentrations that are below the Km value of HIV-1 RT, suggesting that the low dNTP levels kinetically delay HIV-1 reverse transcription in macrophages [8]. Clofarabine is a known RNRI that can deplete endogenous dNTPs in transformed cell lines [21, 22]. Here, we wanted to investigate the extent to which clofarabine depleted endogenous dNTPs in activated CD4+ T cells and macrophages. Specifically, we were interested in whether clofarabine would be effective in the extremely low dNTP environment in macrophages where RNR is not robustly expressed.

We pretreated both cell types, from three healthy donors with two different clofarabine concentrations, 10 nM (below IC50 values for both cell types, Fig. 1b and c) or 300 nM (above IC50, but below CC50 values for both cell types), and methanol extracted the cellular dNTPs 8 h later. To measure the cellular dNTP levels we utilized a single nucleotide primer extension assay that we previously developed [8]. Briefly, this assay uses a 5′ P32 radiolabelled 23-mer primer

(P) annealed to one of four distinct 24-mer templates (T). The single nucleotide overhang on the 24-mer template (A, C, G or T) determines the dNTP to be measured. The template/primer was incubated with extracted cellular dNTPs and purified HIV-1 RT. The increase in radiolabelled 24-mer product indicates that the dNTP specific for the template has been incorporated. In three donors, clofarabine induced at least 50 % reduction of dATP, dCTP and dGTP at 300 nM in activated CD4+ T cells (Fig. 2a) and macrophages (Fig. 2b). As with many RNRIs, dATP levels were the most affected by clofarabine-induced inhibition of RNR. This is possibly due to inefficient dATP synthesis via the salvage pathway, which does not involve RNR and is not affected by RNRIs [32–34]. Consistent with other reports, TTP levels were the least affected in both cell types possibly due to synthesis of TTP from dCMP and dUMP via the salvage pathway [21, 35]. One caveat of this assay is that clofarabine-TP is present in the cells and could be incorporated by HIV-1 RT. The predicted effect of clofarabine-TP incorporation would be an overestimation in the amount of dATP calculated. Despite this potential problem, we saw a strong depletion of dATP in clofarabine-treated cells. This indicates that although the dATP levels may be overestimated we still observe effective RNR inhibition. We also measured the effect of clofarabine on dNTP levels in MAGI cells (a transformed cell line) using a mass spectrometry-based assay, which would be unaffected by clofarabine triphosphate, and saw a similar dNTP depletion profile to that seen in activated CD4+ T cells (Additional file 2: Figure S2A).

As shown in Fig. 2c, the dNTP concentrations in untreated macrophages (M) are already well below the Km of HIV-1 RT, and the clofarabine-induced dNTP reduction (M + clof) would be expected to further delay HIV-1 reverse transcription and inhibit viral infection (as observed in Fig. 1c). However, the dATP concentration in activated CD4+ T cells (T) and in clofarabine-treated T cells (T + clof) remains above the Km value of HIV-1 RT, suggesting that the clofarabine-induced dNTP reduction in T cells should not significantly affect HIV-1 reverse transcription kinetics. Despite this, we do see a reduction in pseudovirus infection in CD4+ T

cells at 300 nM clofarabine treatment (Fig. 1b). One possible explanation is that the toxicity associated with clofarabine treatment may be responsible for the decrease in infection; however, we see potent anti-HIV-1 activity at levels that are not toxic, making this unlikely. Another possibility is it that clofarabine may be acting through an additional mechanism that is not directly related to its RNR inhibition.

Next, we confirmed that the observed clofarabine-induced HIV-1 inhibition (blue lines in Fig. 1b, c) is due to the inhibition of HIV-1 DNA synthesis by employing quantitative PCR to measure a reverse transcription product. Clofarabine treatment led to a decrease in viral DNA synthesis that correlated with a decrease in infection (Fig. 2d). These results are similar to those seen with AZT, a known chain terminator. In contrast, the integrase inhibitor raltegravir (Ralt) decreased infectivity by approximately 80 %, but this loss did not correlate to a reduction in viral DNA synthesis. These results indicate that clofarabine reduces reverse transcriptase-mediated viral DNA synthesis through, at least in part, its inhibition of RNR, which deplete endogenous dNTPs.

Incorporation of clofarabine-TP by reverse transcriptase

Our activated CD4+ T cell data indicated that the clofarabine-induced dNTP depletion would still provide a kinetically favorable environment for reverse transcription. It has been reported that clofarabine triphosphate (clofarabine-TP) is efficiently incorporated into DNA by DNA polymerase α and ε as a dATP analog [21, 22]. Therefore, we tested whether HIV-1 RT can directly incorporate clofarabine-TP into DNA. For this test, clofarabine-TP and purified HIV-1 RT were incubated with a 5′ 32P-labeled 23-mer DNA primer (P) annealed to a 24-mer DNA template containing a single T overhang, which allows RT to incorporate a single clofarabine-TP. As shown in Fig. 3a, a 24-mer extended product (E) was observed in the presence of clofarabine-TP at concentrations as low as 50 nM and the amount of 24-mer produced increased with

increasing concentrations of clofarabine-TP. These results demonstrate that clofarabine-TP is a substrate for HIV-1 reverse transcriptase. HIV-1 RT is known for its high error rate and low fidelity [36, 37], raising the possibility that clofarabine could act as a general purine nucleoside analog. Therefore, we also tested whether clofarabine could be incorporated as a dGTP analog by using a "C" overhang template/primer. In this assay, we did not observe any clofarabine-TP incorporation by HIV-1 RT (data not shown), indicating that clofarabine-TP is incorporated only as a dATP analog, not as a nonspecific purine analog.

Next, we investigated whether the incorporation of clofarabine-TP by HIV-1 RT inhibits primer extension. For this experiment, reactions contained 5′ 32P-labeled 17-mer primer (P) annealed to a 38-mer RNA template (sequence indicated below, Fig. 3 B/D in box), dNTPs (at either the concentrations found in activated T cells, 5 μM, or macrophages, 50 nM), increasing concentrations of clofarabine-TP, and excess HIV-1 RT. As shown in Fig. 3b, when the primer was extended by HIV-1 RT at the T cell dNTP concentrations (5 μM), pausing of DNA synthesis (* in Fig. 3b) was observed only at the highest dose of clofarabine-TP (125 nM). However, at the macrophage dNTP concentrations (50 nM), the RT pausing was observed at much lower clofarabine-TP concentrations, and the full-length extension was completely inhibited at the highest clofarabine-TP concentration (125 nM). As expected, a stall site was observed across from a U site in the template (* in Fig. 3b) indicating that clofarabine-TP was incorporated as an adenosine analog. More interestingly, this stall induced inefficient subsequent dNTP incorporation (] in Fig. 3b), causing RT pausing and eventually blocking full length DNA synthesis. These data indicate that HIV-1 RT can directly incorporate clofarabine-TP and that its incorporation inhibits processive viral DNA synthesis, particularly at the low dNTP environment found in non-dividing macrophages.

There are numerous mutations in RT that render HIV-1 resistant to NRTIs, many of which confer multi-drug resistance. To determine whether these mutations would also confer

resistance to clofarabine, we treated MAGI cells with increasing amounts of clofarabine for 2 h prior to infection with pseudotyped HIV-1 vectors containing a panel of known RT mutations [38]. As shown in Fig. 3C, clofarabine had similar potency against the NRTI-resistant RT mutants as against wild type (NL4-3 MIG). This data could indicate that the primary mechanism of HIV-1 inhibition is RNR inhibition rather than incorporation by RT. However, many mutations in RT that confer resistance to individual NRTIs have no effect on different NRTIs, and some can increase susceptibility to certain drugs. Interestingly, clofarabine's structure is very different from all FDA-approved NRTIs because it has a 3′OH and is not an obligate chain terminator. It is possible that the potency of clofarabine against known NRTI-resistant RTs is due to its differential chemical structure and that clofarabine may work via both mechanisms: RT and RNR inhibition. Selection experiments to develop a clofarabine-resistant strain are planned to further explore this question.

In order to separate the two distinct mechanisms of clofarabine, reduction of dNTPs and direct inhibition of DNA synthesis via incorporation, we biochemically compared the DNA synthesis efficiency of HIV-1 RT at the cellular dNTP concentrations described in Fig. 2c. For this, we extended a 5′ 32P-labeled 17-mer primer (P) annealed to a 38-mer RNA template (sequence indicated in box below Fig. 3b/d) with an equal amount of purified HIV-1 RT protein at three different dNTP concentrations: that found in T cells (T, 5 μM), in macrophages (M, 50 nM) and in macrophages that had been treated with 300 nM clofarabine (M/C, Fig. 3d). This experiment was conducted in the absence of clofarabine so that we could observe the effect of clofarabine induced dNTP reduction on DNA synthesis in the absence of any effects of clofarabine incorporation. As shown in Fig. 3d, HIV-1 RT generated the fully extended product (F) at T cell dNTP concentrations, whereas HIV-1 RT displayed limited primer extension with clear kinetic pausing (*) in the macrophage simulated dNTP concentrations. This RT-pausing was further enhanced at the dNTP concentrations observed in clofarabine treated macrophages, which

ranged from 10 to 40 nM (Fig. 3d). These data recapitulate previous data indicating that the dNTP pools in macrophages have a restrictive effect on reverse transcription [8, 14] and support our hypothesis that a further reduction in dNTPs will lead to a corresponding decrease in HIV-1 viral DNA synthesis. From these biochemical analyses, we suggest that clofarabine induced dNTP depletion in macrophages reduces reverse transcription (Fig. 3d) while also increasing the likelihood of direct incorporation of clofarabine-TP during reverse transcription, which in turn can cause inhibition of processive DNA synthesis (Fig. 3b).

As summarized in Fig. 4, we report that clofarabine, which is converted to clofarabine-DP and clofarabine-TP in cells, can inhibit HIV-1 reverse transcription by two distinct mechanisms: (1) reduction of cellular dNTPs through the inhibition of RNR (by clofarabine-DP and clofarabine-TP) and (2) direct incorporation of clofarabine-TP by HIV-1 RT and inhibition of viral DNA synthesis. Importantly, the inhibitory impact of clofarabine imposed by these two mechanisms becomes more effective in non-dividing macrophages because this viral target cell type is less sensitive to the toxic effects of clofarabine. In addition, macrophages maintain limited dNTP pools, in part due to the dNTPase activity of host protein SAMHD1. Interestingly, many nucleoside analogs are not substrates for SAMHD1, which increases nucleoside reverse transcriptase inhibitor (NRTI) efficacy in macrophages [39]; however, it was recently published that clofarabine-TP is degraded by SAMHD1 in vitro [25]. Despite this, clofarabine was still able to reduce dNTPs and restrict viral replication efficiently in macrophages.

Our previous publication reported that clofarabine is threefold more effective against HIV-2 than HIV-1 [23]. This result is surprising in light of our current report that clofarabine exerts its antiviral effect at least in part through reducing dNTP levels: the HIV-2 protein Vpx counteracts SAMHD1 and promotes retroviral replication in macrophages by elevating cellular dNTPs, and therefore would be expected to be more resistant to clofarabine than HIV-1, which does not contain Vpx. However, the previous work on HIV-2 was performed in U373-MAGI-

CXCR4CEM cells, a transformed cell line derived from a human glioblastoma, which have very limited SAMHD1 expression, abundant dNTPs and are highly permissive to HIV-1 infection. This indicates that the difference seen between HIV-1 and HIV-2 inhibition by clofarabine in the previous report is likely not Vpx or dNTP related but more likely related to differences in RT fidelity. Comparing the effect of clofarabine on HIV-1 and HIV-2 in primary human macrophages would be interesting, but we believe it would be difficult to explain the outcome mechanistically due to the SAMHD1-mediated hydrolysis of clofarabine-TP (31). Degradation of SAMHD1 by Vpx would lead to an increase in both dNTP and clofarabine-TP levels complicating the expected experimental outcome of the potency of clofarabine. A dual RNRI/NRTI that is resistant to SAMHD1 hydrolysis would be expected to have significantly higher efficacy against HIV-1 than does clofarabine, and would be a much better tool to study the effects of Vpx on RNR inhibition.

Any suggestion of using an RNRI as a clinical treatment for HIV-1 runs into the specter of hydroxyurea, an RNRI which failed clinically as an anti-HIV drug due primarily to its accentuation of NRTI toxicity [40]. Given the self-potentiation inherent to clofarabine's dual mechanisms of action, potentiation of toxicity would be a serious risk; however, there are significant differences between the two RNRIs. Hydroxyurea and clofarabine differ greatly in structure and in the mechanism of RNR inhibition. Clofarabine has been used as a second line cancer treatment since its FDA approval in 2004 and recent trials have had positive results using it as a first line cancer treatment that can be administered orally [41, 42]. Any use of clofarabine as an HIV drug would require further drug interaction studies to establish the safety of clofarabine both alone and with commonly used combination therapies. The current study was designed to study the mechanism of action and toxicity of clofarabine as a model for dual RNR/HIV-1 RT inhibitors in primary human target cells. We propose that clofarabine be used as a base molecule for further drug design studies. A derivative that shows resistance to SAMHD1

hydrolysis while maintaining the activity and low toxicity of clofarabine in macrophages would be of great interest. This would increase drug efficacy by reducing the natural dNTP competition and ensure that drug concentrations remain high for increased incorporation. In conclusion, our experimental observations of cellular dNTP level reduction by clofarabine (below Km value of HIV-1 RT) and more effective incorporation of clofarabine-TP at these lowered dNTP concentrations support that clofarabine can be a model agent for dual function anti-HIV agents.

Methods

Cell lines, plasmids, and chemicals

The 293T cell line was obtained from the American Type Culture Collection (Manassas, VA) while U373-MAGI-CXCR4CEM (MAGI) cells were obtained from Dr. Michael Emerman through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH [43, 44]. The HIV plasmid pNL4-3 MIG has been previously described [24]. RT resistant mutants were created as previously described [38] and subcloned into pNL4-3 MIG. The VSV-G envelope plasmid, pHCMV-VSVG, was provided by J. Burns (University of California, San Diego). Clofarabine was purchased from Carbosynth (Berkshire, UK). The following reagents were obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Raltegravir (Cat # 11680) from Merck & Company, Inc. and zidovudine.

Cell culture

293T and MAGI cells were maintained as previously described [45]. Primary human monocytes and CD4+ T cells were isolated from peripheral blood buffy coats by positive selection using CD14 or CD4 beads (Miltenyi Biotec, San Diego, CA), as previously described [46], and maintained in RPMI medium with 10 % FCS and penicillin/streptomycin. CD14+ monocytes were matured to macrophages using 5 ng/mL human GM-CSF for 7 days (Miltenyi

Biotec). CD4+ T cells were activated with 5 µg/mL phytohemagglutinin (Sigma-Aldrich) and 20 units/mL IL2 (Miltenyi Biotec) for 1 day, then 20 units/mL IL2 alone for 5 days.

Production of viral stocks

293T cells were co-transfected with pNL4-3 MIG and pHCMV-VSVG using linear polyethylenimine from Polysciences, Inc. (Warrington, PA) as previously described [47]. Virus was harvested at 48 and 72 h post-transfection and used immediately or frozen at −80 °C.

Drug treatments and infections

MAGI cells were infected as described previously [45]. Cells were treated with clofarabine for 2 h prior to infection with viral supernatant. Cells were harvested for analysis 48–72 h after infection and analyzed with a BD Biosciences LSRII flow cytometer. Vehicle treated cells had 15–35 % infection. Primary cells were treated with clofarabine for 8 h, washed twice with PBS and then infected with viral supernatant. To achieve the same infectivity, MDMs were infected with five times the amount of viral supernatant than T cells. Monocyte derived macrophages (MDM) were collected 5 days post-infection and activated CD4+ T cells were collected 3 days post-infection for analysis via flow cytometry. Data analysis was performed using FloJo and Prism 6. IC50 and CC50were determined using GraphPad Prism nonlinear fit analyses; log (inhibitor) versus response–variable slope.

Cell proliferation and cytotoxicity

MDMs and T cells were treated with clofarabine for 8 h, washed twice with PBS and maintained in media 5 or 3 days, respectively (same treatment as infection protocol). The XTT assay from ATCC was used as per the manufacturer's protocol. The optimized assay incubation time for both cell types was 5 h. For additional measures of toxicity, including trypan blue

exclusion and cell size, cells were treated the same as above and analyzed using the Vi-Cell Counter (Beckman Coulter).

Real-time qPCR of RT products

Real time qPCR was performed essentially as previously described [48]. MAGI cells (150,000 per well) were plated on a six-well plate. Twenty-four hours later, cells were treated with the indicated drug for 2 h prior to infection. As a control, an aliquot of virus was heat inactivated for 30 min at 95 °C. Eighteen hours after infection, cells were harvested with trypsin; half were re-plated for the analysis of infection, and DNA was isolated from remaining cells using HighPure PCR Template Preparation Kit (Roche, Basel, Switzerland). Quantitative PCR (qPCR) mixtures contained 4 μL of eluted DNA using iTaq Universal SYBR Green Supermix according to the manufacturer's suggestions (BioRad, Hercules, CA). Primers for 18S rRNA were used to normalize sample-to-sample variation. The primers used to detect late reverse transcription (RT) products were 5′TGTGTGCCCGTCTGTTGTGT (forward) and 5′GAGTCCTGCGTCGAGAGAGC (reverse). The primers used to detects 18S rRNA were 5′GTAACCCGTTGAACCCCATT (forward) and 5′CCATCCAATCGGTAGTAGGG (reverse). The conditions for amplification were 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s.

Synthesis of clofarabine triphosphate

Clofarabine triphosphate was synthesized as previously described [49] with minor modifications. In separate round-bottom flasks, clofarabine (0.1 g, 1.0 eq.) and tributylammonium pyrophosphate (0.361 g, 2.0 eq.) were dried under high vacuum for 1 h at ambient temperature. Throughout the entire experiment, the reaction was maintained under an argon atmosphere. Clofarabine (0.1 g, 1.0 eq.) was dissolved in anhydrous dioxane (0.9 mL, 3 mL/mmol) and

anhydrous pyridine (0.3 mL, 1 mL/mmol). The flask was cooled with an ice bath. A solution of 2-chloro-4H-1,3,2- benzodioxaphosphorin-4-one (80 mg, 1.2 eq.) in anhydrous dioxane (0.4 mL, 1 mL/mmol) was added and the mixture was then stirred at room temperature for 15 min. A solution of tributylammonium pyrophosphate (361 mg, 2.0 eq.) in anhydrous DMF (0.7 mL, 1 mL/mmol) was added to the mixture, prior to quick addition of tributylamine (0.4 mL, 5.0 equiv). The mixture was stirred for 15 min at room temperature. A solution of iodine (1 % solution in pyridine/water, 9:1) was then added dropwise until the permanent brown color of iodine persisted, and the mixture was stirred for 20 min. The excess of iodine was quenched with a 5 % aqueous solution of Na2S2O3. The reaction mixture was evaporated to dryness under vacuum, dissolved in 25 % ammonia solution, and stirred for 1 h at room temperature. The reaction was monitored by TLC (Rf 0.1 isopropanol/aqueous ammonia = 1/1 and MS). The reaction mixture was concentrated under reduced pressure and resulting crude product was purified on a short pad of silica gel with a gradient of isopropanol/aqueous ammonia 8/2 to 1/1 affording the desired 5′-triphosphate ammonium salt, 21 % yield (75 % pure with diphosphate as an impurity, determined by 31P NMR). The synthesized nucleoside 5′-triphosphate was confirmed by 1H NMR, 31P NMR, and HR-MS analyses consistent with published anaalytical data for clofarabine 5′-triphosphate [19].

HIV-1 RT purification

HIV-1 (NL4-3) RT homodimer with a hexahistidine tag was expressed using an overexpression system in BL21 *E. coli* [50, 51], and purified using Ni2+ chelation chromatography as described previously [8, 51].

dNTP quantification

dNTPs from primary cells were extracted and quantified using the protocol previously described by Diamond et al. [8]. Briefly, this assay uses a 5′ P32 radiolabelled 23-mer primer (P) annealed to one of four distinct 24-mer templates (T). The single nucleotide overhang on the 24-mer template (A, C, G or T) determines the dNTP to be measured. The template/primer was incubated with extracted cellular dNTPs and purified HIV-1 RT for 5 min at 37° and then quenched with 40 mM EDTA and 99 % (vol/vol) formamide at 95 °C for 2 min. The reactions were resolved on a 20 % urea-PAGE gel (American Bio Sequel NE reagent) and imaged using Pharos FX molecular imager (Biorad). The increase in radiolabelled 24-mer product indicates that the dNTP specific for the template has been incorporated. MAGI cells were treated with 100 or 300 nM clofarabine for 8 h, then harvested, counted, resuspended in 60 % methanol, and stored at −20 °C for 18 h. Samples were then vortexed, heated to 95 °C for 3 min, and centrifuged at 16,000×g for 5 min. Supernatant was transferred to new tubes and dried in an Eppendorf Vacufuge. Samples were then stored at −80 °C.

To perform LC–MS/MS analysis, dried extracts were reconstituted in water at a concentration of 1 million cells per 100 µL. For each reconstituted sample, a 50 µL aliquot of resuspension was added to an Eppendorf tube containing 50 µL of internal standard (10 µM 5-iodo-dCTP in water), and then diluted with 100 µL water. The samples were then centrifuged at 14,000 rpm for 5 min at 4 °C. LC–MS/MS was used to determine levels of TTP, dGTP, dCTP and dATP in the supernatants following a previous published method [52] with minor modifications. The LC–MS/MS system consists of an AB Sciex QTrap 5500 mass spectrometer and an Agilent 1260 Infinity HPLC. The chromatographic separation of analytes was achieved using a Thermo Scientific Hypercarb column (100 × 3 mm, 5 µm). The two eluents were: (A) 0.5 % diethylamine in water, pH adjusted to 10 with acetic acid; and (B) 50 % acetonitrile in water. The mobile phase was delivered at a flow rate of 0.5 mL/min using stepwise gradients of A and B: 0–20 min, 0–25 % B (v/v); 20–28 min, 25–50 % B (v/v); 28–28.5 min, 50–95 % B (v/v);

28.5–30.5 min, 95–95 % B (v/v); 30.5–31 min, 95–0 % B, (v/v); 31–39 min, 0–0 % B (v/v). Only eluate from 10-30 min was diverted into the mass spectrometer for analysis. MS/MS detection of the analytes was conducted using an ESI ion source with MRM detection in negative mode. The curtain gas was set at 20 psi. The ionspray voltage was set at −4500 V, and the temperature at 650 °C. The nebulizer gas (GS1) and turbo gas (GS2) were both set at 45 psi.

In vitro clofarabine triphosphate incorporation assay

This single nucleotide extension assay was modified from a previously described assay [51]. A 5′32P-labeled DNA 18-mer (5′-GTCCCTCTTCGGGCGCCA-3′) was annealed to a 19-mer DNA template (3′-CAGGGAGAAGCCCGCGGTG-5′) at a 1:2 ratio. Extension from an 18-mer to 19-mer indicates that clofarabine triphosphate has been incorporated by HIV-1 reverse transcriptase. 20 μL reactions contained 200 fmol template/primer, 2 μL clofarabine triphosphate at concentrations indicated or 50 μM of dNTPs for the positive control, 4 μL of purified RT (HIV-1 NL4-3), 25 mM Tris–HCl, pH 8.0, 2 mM dithiothreitol, 100 mM KCl, 5 mM MgCl2, and 10 μM oligo(dT). Reactions were incubated at 37 °C for 5 min and then quenched with 10 μL of 40 mM EDTA and 99 % (vol/vol) formamide at 95 °C for 2 min. The reactions were resolved on a 20 % urea-PAGE gel (American Bio Sequel NE reagent) and imaged using Pharos FX molecular imager (Biorad).

Primer extension assay

An HIV-1 RT primer extension assay was performed as previously described with slight modifications [53]. A 5′ 32P-labeled 17-mer DNA primer (5′-CGCGCCGAATTCCCGCT-3′) was annealed to a 40-mer RNA template (5′-AAGCUUGGCUGCAGAAUAUUGCUAGCGGGAAUUCGGCGCG-3′) in the presence of 100 mM NaCl, 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA. 20 μL Reaction mixtures contained 10

nM template-primer, 4 µL of purified HIV-1 RT, 5 µM or 50 nM of all four dNTPs (ThermoScientific), 12.5 mM Tris–HCl (pH 7.5), 12.5 mM NaCl, 2.5 mM MgCl2 and 20 µM oligo(dT). Reactions were initiated upon addition of RT and incubated at 37 °C for 1 h (for Fig. 3b) and 5 min (for Fig. 3d). Reactions were terminated with 10 µL of 40 mM EDTA, 99 % formamide and the products were resolved on a 14 % urea-PAGE gel (American Bio Sequel NE reagent) and imaged using Pharos FX molecular imager (Biorad) and analyzed using ImageLab software.

Authors' contributions

MBD performed all primary cell experiments and biochemical assays. MER did all MAGI cell experiments and proviral DNA synthesis analysis. LB and SP designed and synthesized clofarabine triphosphate. JOM produced RT mutant vectors. JX performed MS/MS-based dNTP assay. CLC contributed to experimental design. BK and LM conceived experiments and prepared manuscript. All authors read and approved final manuscript.

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Competing interests

The authors declare that they have no conflicts of interest with the contents of this article.

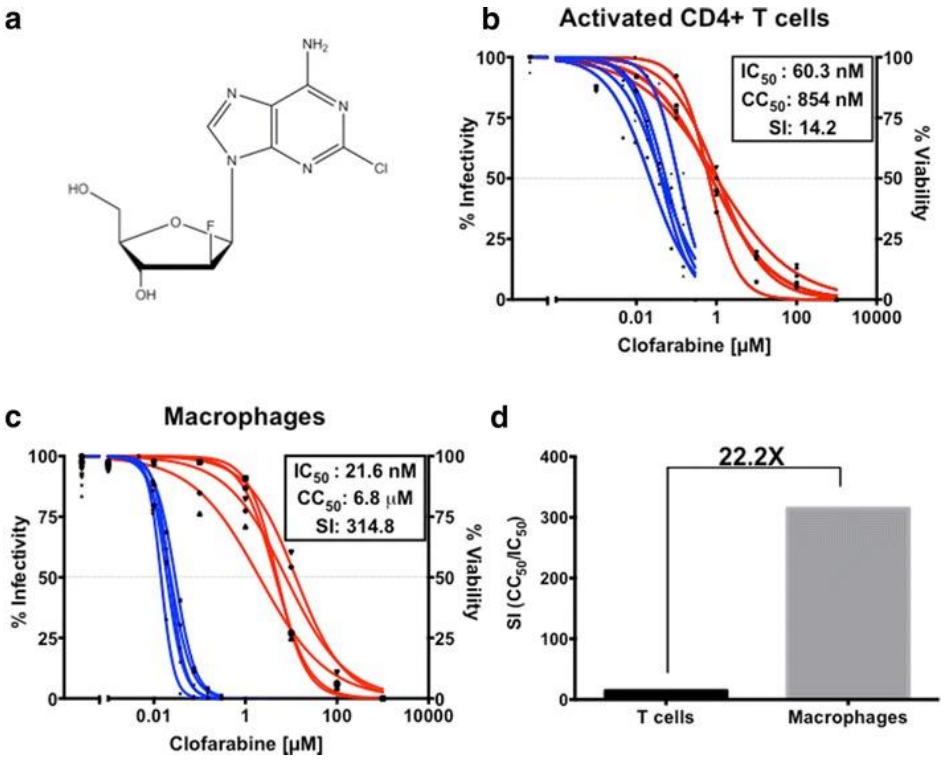

Figure 1. Anti-HIV-1 activity of clofarabine in primary human activated CD4+ T cells and monocyte derived macrophages. **a** The structure of clofarabine. **b** Clofarabine inhibition (*blue lines*) and cytotoxicity (*red lines*) on activated CD4+ T cells of 5 healthy donors. Cells were treated with increasing concentrations of clofarabine for 8 h, washed with PBS, and then infected with pseudotyped HIV-1, (inhibition) or cultured with media (cytotoxicity). Analysis was conducted at 72 h post-infection via flow cytometry (inhibition) or XTT assay (cytotoxicity). The IC50is 60.3 nM with a 95 % confidence interval (95 % CI) of 24.1–96.5 nM; the CC50 is 854.2 nM with a 95 % CI of 712.6–995.8 nM. **c** The clofarabine inhibition (*blue lines*) and cytotoxicity curves (*red lines*) for monocyte-derived macrophages of 5 healthy donors. Macrophages were treated as described for T cells except analysis was at 5 days post infection. IC50 = 21.6 nM (95 % CI 17.4–25.8 nM): CC50 = 6.8 µM (95 % CI 3.2–9.4 µM). **d** Selectivity Index (SI) difference between activated CD4+ T cells and macrophages. SI values were determined by dividing the Average CC50 of five donors by the average IC50.

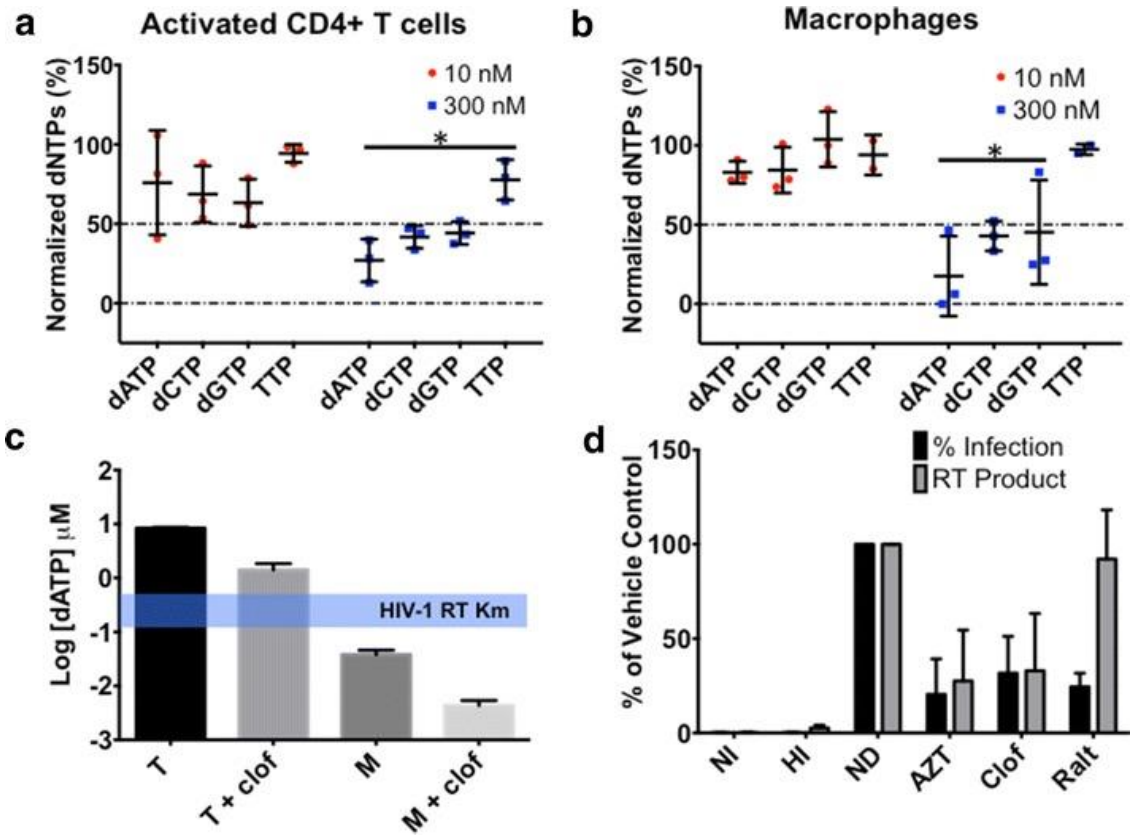

Figure 2. Clofarabine induced depletion of cellular dNTPs and inhibition of reverse transcription. Effect of clofarabine on cellular dNTP levels in primary activated CD4+ T cells (**a**) and macrophages (**b**) from three healthy donors. Activated CD4+ T cells and macrophages were treated with the indicated concentration of clofarabine for 8 h, washed with PBS, and dNTPs were extracted for analysis. dNTP levels are expressed as a percentage of the vehicle control (DMSO). Each value represents an individual donor with mean ± SD indicated. *p < .05 compared to vehicle control (multiple t test with Holm-Sidak post hoc test). **c** Cellular dATP concentrations of untreated and clofarabine (300 nM) treated T cells and macrophages from three healthy donors. Measured dATP levels were converted to cellular dATP concentrations as previously described, and are expressed as mean ± SD). *Blue bar* indicates the Km range of HIV-1 RT. T: untreated T cells, T + clof: T cells + 300 nM clofarabine, M: untreated macrophages, M + clof: macrophages + 300 nM clofarabine. **d** Clofarabine effect on HIV-1 proviral DNA

synthesis. MAGI cells were incubated with DMSO (NI, HI, ND), 200 nM AZT, 300 nM clofarabine (Clof), or 50 nM raltegravir (Ralt) for 2 h prior to infection with pseudotyped HIV-1. Infection (*black bars*) was determined at 48 h post-infection, and reverse transcription (*grey bars*) efficiency was determined at 18 h post-infection. Data represent mean ± SD from three independent experiments and are expressed as percentage of vehicle control (DMSO, 100 %). *NI* no infection, *HI* heat inactivated virus, *ND* no drug

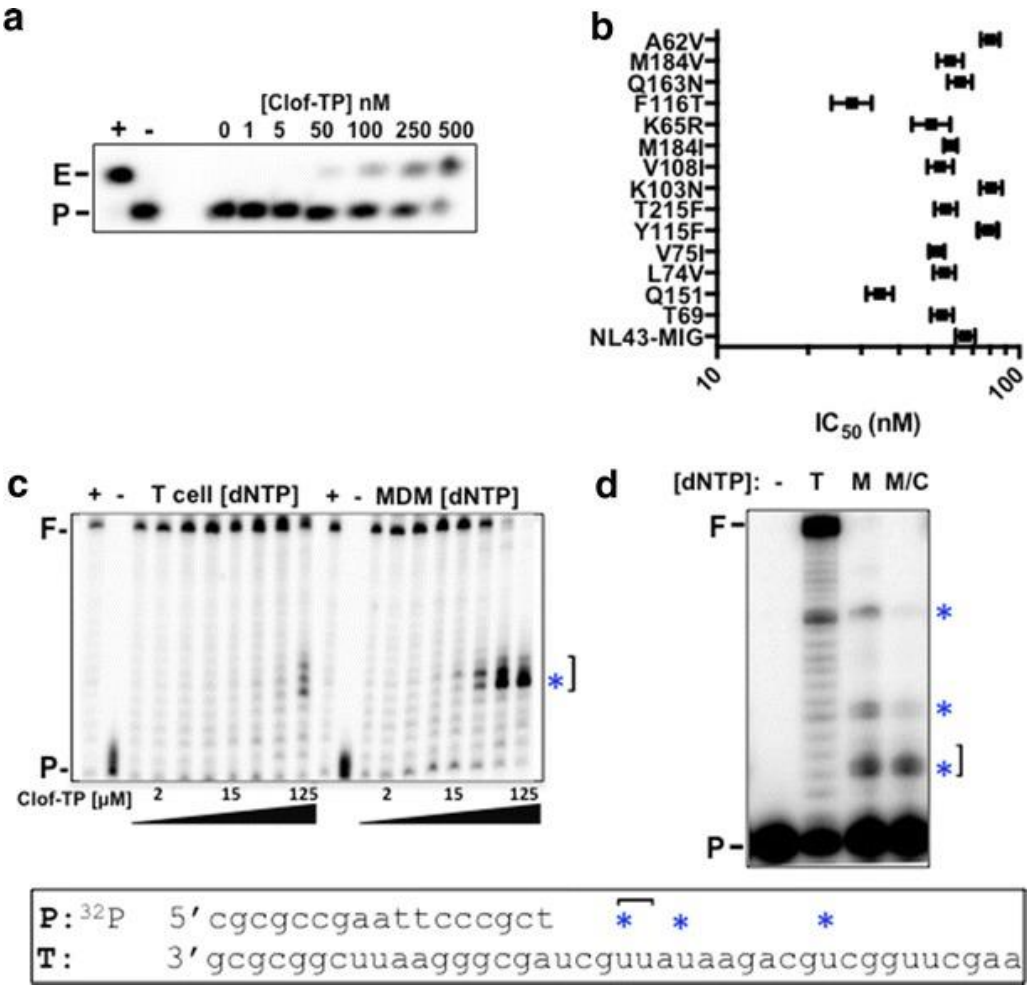

Figure 3. Biochemical examination of the dual mechanism of clofarabine. **a** Direct clofarabine-TP incorporation by HIV-1 RT. A 5′ 32P-labeled 23-mer DNA primer (P) annealed to a 24-mer DNA template with a single T overhang was incubated with HIV-1 RT and increasing concentrations of clofarabine-triphosphate (Clof-TP). E, Extended product; +, 50 μM dATP positive control; −, no dATP control. **b** Effect of clofarabine-TP incorporation on DNA synthesis. A 5′ 32P-labeled 17-mer DNA primer (P) annealed to a 38-mer RNA template was extended by HIV-1 RT with fixed dNTP concentrations found in either activated T cells (T cell, 5 μM) or monocyte-derived macrophages (MDM, 50 nM) with increasing concentrations of clofarabine-TP (two-fold dilutions starting at 125 μM). F, Fully extended product; +, 50 μM dNTP positive control; −, no dNTP control. *Blue asterisks* (*) indicates the clofarabine-TP incorporation site followed by kinetic pauses (]). **c** Clofarabine inhibition of NRTI-resistant RT mutants. MAGI

cells were treated with increasing concentrations of clofarabine for 2 h prior to infection with Vsvg-pseudotyped HIV-1 containing mutations in RT. Flow cytometry analysis for infected cells was conducted at 48–72 h post-infection. IC50 values and 95 % confidence intervals are shown. NL4-3 MIG: wild-type HIV-1 RT, Q151: A62V, V75I, F77L, F116Y and Q151M, T69: M41L, A62V, T69S, K70R, T215Y and serine–serine insertion between 69 and 70. **d** Biochemical simulation of HIV-1 RT activity at dNTP concentrations found in cells with and without clofarabine treatment. A 5′ 32P-labeled 17-mer DNA primer (P) annealed to a 38-mer RNA template (shown in *box*) was extended using an equal amount of purified HIV-1 RT protein with dNTP concentrations found in T cells (T, 5 μM), macrophages (M, 50 nM) or macrophages treated with 300 nM clofarabine (M/C, 10 nM dATP, 28 nM dCTP, 28 nM dGTP, 40 nM TTP). *Blue stars* (*) indicate kinetic pause sites, F, fully extended 38 bp product; −, no dNTP control

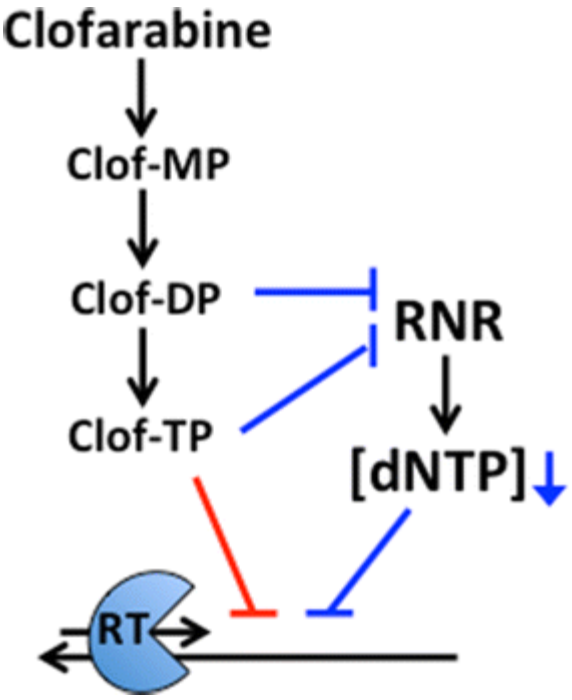

Figure 4. Model for the anti-HIV-1 dual action mechanisms of clofarabine in macrophages. Clofarabine di- and triphosphate inhibit RNR to reduce dNTP levels, leading to the kinetic suppression of HIV-1 reverse transcription (*blue*). Clofarabine triphosphate is also directly incorporated by HIV-1 RT, inhibiting extension (*red*)

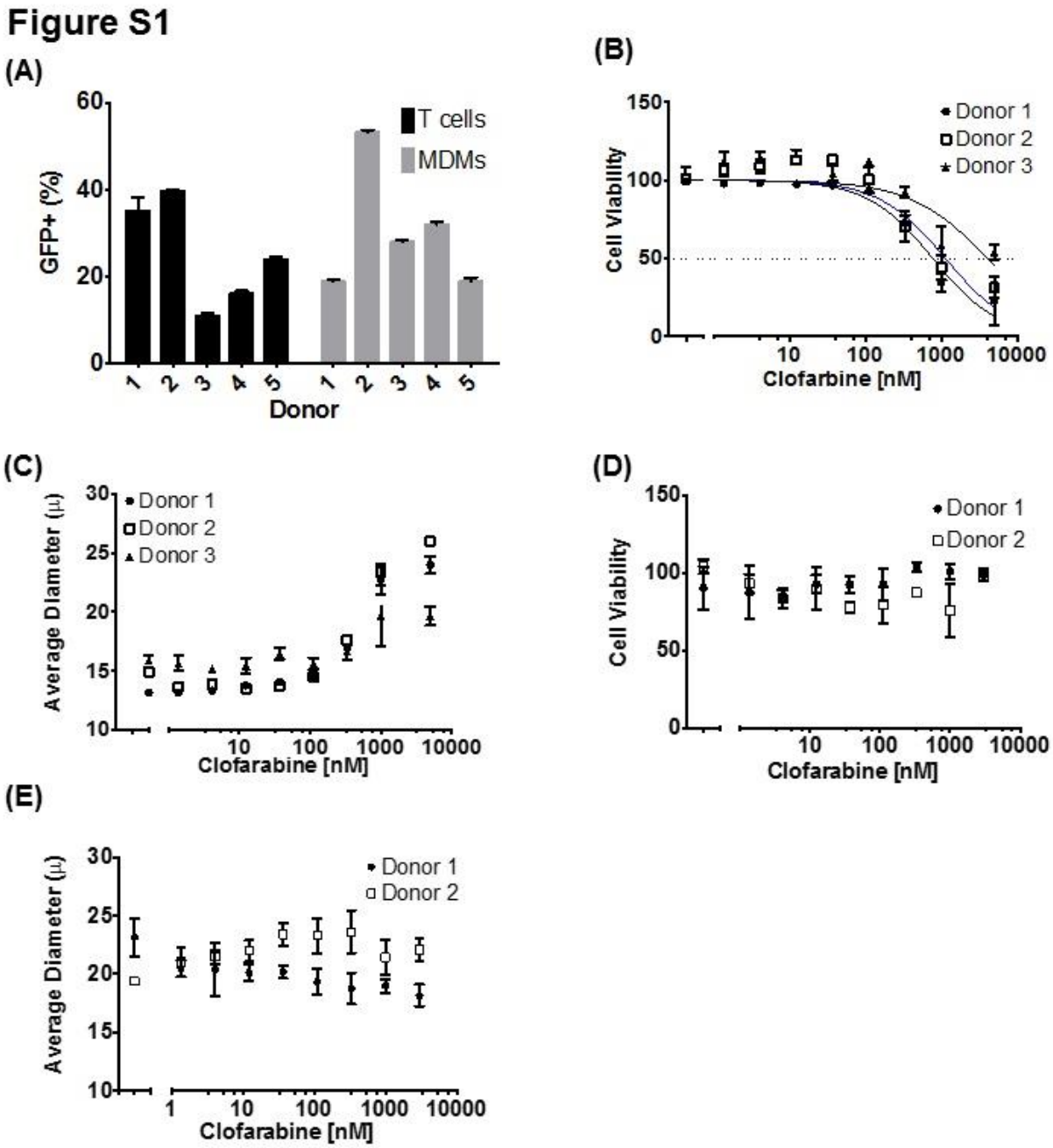

Figure S1. Infectivity and toxicity in T cells and macrophages. **(A)**Activated CD4+ T cells and macrophages were infected with pseudotyped HIV-1. In order to achieve similar levels of infection more pseudovirus was used to infect macrophages (5X more virus used). Individual donor infectivity is shown with technical triplicates shown as standard error (mean with standard deviation). **(B)** Cell viability in activated CD4+ T cells. Cells were treated with varying amounts

of clofarabine for 8 h, washed in PBS and maintained in media for 72 h. Cell viability was determined by exclusion of trypan blue indicating membrane integrity by the Vi-cell counter. **(C)** Cell size of activated CD4+ T cells treated with clofarabine. Cells were treated with varying amounts of clofarabine for 8 h, washed in PBS and maintained in media for 72 h. Cells were counted and cell size was determined using the Vi-Cell counter. **(D)** Cell viability in macrophages. Cells were treated with varying amounts of clofarabine for 8 h, washed in PBS and maintained in media for 5 days. Cell viability was determined by exclusion of trypan blue indicating membrane integrity by the Vi-cell counter. **(E)** Cell size of activated CD4+ T cells treated with clofarabine. Cells were treated with varying amounts of clofarabine for 8 h, washed in PBS and maintained in media for 5 days. Cells were counted and cell size was determined using the Vi-Cell counter.

Figure S2

(A)

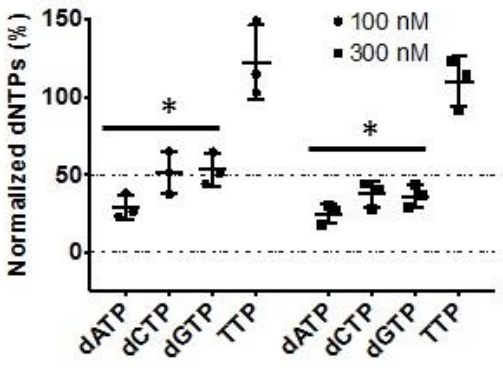

Figure S2.Clofarabine induced depletion of cellular dNTPs in MAGI cells. MAGI cells were treated with 100 nM (~IC50) and 300 nM (~IC90) clofarabine for eight hours, washed with PBS, and dNTPs were methanol extracted and analyzed by LC–MS/MS. Data shown represents mean ± SD of three independent experiments, and is expressed as a percentage of vehicle control. * = $p < .05$ compared to vehicle control (multiple t test with Holm-Sidak post hoc test).

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CHAPTER III: 5-Azacytidine Enhances the Mutagenesis of HIV-1 by Reduction to 5-Aza-2'-Deoxycytidine

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M.Daly performed all biochemical experiments, specifically figure 4.

Abstract

5-Azacytidine (5-aza-C) is a ribonucleoside analog that induces the lethal mutagenesis of human immunodeficiency virus type 1 (HIV-1) by causing predominantly G-to-C transversions during reverse transcription. 5-Aza-C could potentially act primarily as a ribonucleotide (5-aza-CTP) or as a deoxyribonucleotide (5-aza-2=-deoxycytidine triphosphate [5-aza-dCTP]) during reverse transcription. In order to determine the primary form of 5-aza-C that is active against HIV-1, Illumina sequencing was performed using proviral DNA from cells treated with 5-aza-C or 5-aza-dC. 5-Aza-C and 5-aza-dC were found to induce highly similar patterns of mutation in HIV-1 in terms of the types of mutations observed, the magnitudes of effects, and the distributions of mutations at individual sequence positions. Further, 5-aza-dCTP was detected by liquid chromatography–tandem mass spectrometry in cells treated with 5-aza-C, demonstrating that 5-aza-C was a substrate for ribonucleotide reductase. Notably, levels of 5-aza-dCTP were similar in cells treated with equivalent effective concentrations of 5-aza-C or 5-aza-dC. Lastly, HIV-1 reverse transcriptase was found to incorporate 5-aza-CTP in vitro at least 10,000-fold less efficiently than 5-aza-dCTP. Taken together, these data support the model that 5-aza-C enhances the mutagenesis of HIV-1 primarily after reduction to 5-aza-dC, which can then be incorporated during reverse transcription and lead to G-to-C hypermutation. These findings may have important implications for the design of new ribonucleoside analogs directed against retroviruses.

Background

RNA viruses exhibit high mutation rates and have been postulated to replicate near the error threshold—the maximal mutation rate compatible with the maintenance of genetic information (1, 2). Thus, these viruses may be particularly sensitive to small molecules that promote viral mutations, an antiviral strategy called lethal mutagenesis (3). Lethal mutagenesis has been pursued as a potential antiviral approach for many different RNA viruses (4). Most small-molecule candidates for lethal mutagenesis identified thus far have been nucleoside analogs with

altered base-pairing properties. These nucleoside analogs base pair promiscuously due to ionization, structural rearrangement, tautomerization, or conformational flexibility (5). Alternatively, small molecules can be used to promote viral mutagenesis by leveraging host nucleic acid-editing enzymes that are part of the innate immune response. For example, molecules have been identified that block the degradation of APOBEC3 enzymes by the human immunodeficiency virus type 1 (HIV-1) accessory protein Vif (6–9). These molecules ultimately promote the incorporation of APOBEC3 proteins into virions, resulting in lethal G-to-A hypermutation during the next cycle of replication. The ribonucleoside analog 5-azacytidine (5-aza-C) reduces the infectivity of HIV-1 by inducing lethal mutagenesis (10). 5-Aza-C is active during both the early and late phases of viral replication, reflecting incorporation during both reverse transcription and the transcription of viral genomic RNA, respectively. When added during the late phase of viral replication, 5-aza-C induces primarily C-to-G transversions in HIV-1. In contrast, during the early phase of replication, 5-aza-C induces primarily G-to-C transversions in the virus. These G-to-C transversions are thought to be caused by the incorporation of 5-aza-C into minus-strand viral DNA, followed by the hydrolysis of 5-aza-C and its deformylation into ring-opened remnants (10). These ring-opened remnants can then mispair with deoxycytidine during plus-strand synthesis, leading to the fixation of G-to-C transversions in proviral DNA. However, it remains possible that 5-aza-C hydrolysis products are directly incorporated by HIV-1 reverse transcriptase (RT) as well. Notably, 5-aza-C is closely related to 5-aza-2=-deoxycytidine (5- aza-dC), another nucleoside analog that has been explored for the lethal mutagenesis of HIV-1 (11, 12); the primary difference is that 5-aza-dC is much more potent than 5-aza-C and likely is incorporated only into viral DNA. Two different mechanisms could account for the antiviral activity of 5-aza-C during the early phase of replication: First, 5-aza-C could be incorporated during reverse transcription primarily as a deoxyribonucleotide (i.e., as 5-aza-2=-deoxycytidine 5=-triphosphate [5-aza-dCTP]). For this to occur, the cellular enzyme ribonucleotide reductase (RNR) would have to first convert 5-aza-C (in its diphosphate form) to

5-aza-dC, which could then be phosphorylated to form 5-aza-dCTP. Notably, one previous study has demonstrated that 10 to 20% of 5-aza-C is reduced to 5-aza-dC by RNR (13), suggesting that 5-aza-dCTP would likely be available for incorporation during reverse transcription. However, the reduction of 5-aza-C to 5-aza-dC has not yet been demonstrated in cell types for which antiviral activity has been reported. In further support of this possibility, HIV-1 RT has been shown to selectively exclude ribonucleotides by using a residue (Y115) that acts as a steric gate (14–16). Alternatively, 5-aza-C might be incorporated directly as a ribonucleotide (i.e., as 5-azaCTP) during reverse transcription. Notably, HIV-1 RT has been found to incorporate significant levels of endogenous ribonucleotides when the levels of deoxyribonucleotides are very low (resulting in high nucleoside triphosphate [NTP]/deoxynucleoside triphosphate [dNTP] ratios), as they are in macrophages (17, 18). Previous studies have found that high concentrations of 5-aza-C are required to elicit antiviral activity in cell culture (10), potentially skewing the NTP/dNTP ratio enough to allow for significant 5-aza-CTP incorporation. However, these findings could also re- flect the inefficient reduction of 5-aza-C to 5-aza-dC. In order to determine the primary form of 5-aza-C that is active during HIV-1 reverse transcription, Illumina high-throughput sequencing was performed to compare viral mutagenesis in the presence of 5-aza-C with that in the presence of 5-aza-dC. 5-Aza-C and 5-aza-dC caused similar levels of G-to-C and C-to-G transversions in HIV-1. Further, G-to-C and C-to-G transversions at individual sequence positions were distributed in highly similar patterns for 5-aza-C and 5-aza-dC. In addition, 5-azadCTP was detected in cells treated with 5-aza-C, indicating that 5-aza-C was reduced to 5-aza-dC by RNR. Importantly, 5-azadCTP levels were similar in cells treated with equivalent effective concentrations of 5-aza-C or 5-aza-dC. Lastly, 5-aza-CTP was incorporated in vitro by HIV-1 RT, but much less efficiently than 5-aza-dCTP. Overall, the data support the conclusion that 5-aza-C enhances HIV-1 mutagenesis and diminishes HIV-1 infectivity primarily by acting as 5-aza-dCTP, which may have important implications for the design of antiretroviral ribonucleoside analogs.

Material and Methods

Plasmids, cell lines, and reagents. For single-cycle infections, viral stocks were produced by cotransfecting the HIV-1 envelope-deficient vector pNL4-3 MIG (19), which expresses mCherry and enhanced green fluorescent protein (EGFP), and pHCMV-G, a kind gift from J. Burns (University of California, San Diego). Viral stocks were produced in human embryonic kidney (HEK 293T) cells from the American Type Culture Collection (Manassas, VA). Viral infections were performed in U373- MAGI-CXCR4CEM cells, obtained from Michael Emerman through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (20). 5-Aza-C was purchased from Sigma-Aldrich (St. Louis, MO), while 5-aza-dC divalerate (referred to below simply as 5-aza-dC), a stabler prodrug form of 5-aza-dC, was synthesized by the Center for Drug Design a the University of Minnesota as described previously (21). 5-Aza-CTP and 5-aza-dCTPwere purchased from American Advanced Scientific (College Station, TX) and Jena Bioscience (Jena, Germany), respectively. All drugs were dissolved in the appropriate solvent and were stored in aliquots at 20°C. Production and titration of viral stocks for Illumina sequencing. HIV-1 stocks for Illumina sequencing were produced by cotransfecting 10 ug of pNL4-3 MIG and 1 ug of pHCMV-G per 10-cm plate of 293T cells. Transfections were carried out using the polyethyleneimine (PEI) method, as described previously (19). Viral supernatants were collected 48 h posttransfection, treated with 10 U/ml of DNase I for 2 h at 37°C to reduce plasmid carryover from transfections, and frozen in aliquots at 80°C. The titers of viral stocks were determined by infecting U373-MAGI cells (31,250 cells/well in 24-well plates) with volumes of virus ranging from 1.25 to 40 (2-fold series). Viral infectivity was determined by performing flow cytometry on cells collected 72 h postinfection. Viral titers were determined by plotting the volume of virus against the percentage of infected cells (i.e., cells expressing mCherry and/or EGFP). Determination of viral mutation frequencies by Illumina sequencing. Samples were prepared for Illumina amplicon sequencing as described previously (22), but with several minor

modifications. Briefly, dimethyl sulfoxide (DMSO) (for the no-drug control), 5-aza-C (75% effective concentration [EC75], 260 uM), or 5-aza-dC (EC75, 3.75 uM) was added to U373-MAGI cells (1 million cells/sample) 2 h before infection. The cells were then infected at a multiplicity of infection (MOI) of 1.0 by adding the appropriate volume of NL4-3 MIG-VSVG. The medium was replaced 24 h postinfection, and cells were collected 72 h postinfection. Genomic DNA was purified from cell pellets, and plasmid carryover (from transfections) was then assessed by quantitative PCR (qPCR). Levels of plasmid carryover were determined by dividing the starting quantity of the ampicillin resistance gene by the starting quantity of HIV-1 vif, using previously published primers (22). The average level of plasmid carryover was found to be 0.4% (range, 0.1 to 0.7%). Five small (160- to 170-bp) amplicons (Gag, Pol, Vif, Env, Nef) were then prepared from each sample by PCR, using the primers listed in Table 1. PCRs were also performedwith the purified plasmid to determine the level of background error due to PCR and Illumina sequencing. For each sample, all amplicons were gel purified, quantified, and pooled in an equimolar fashion to normalize coverage between amplicons. Sequencing libraries were prepared from each sample using the TruSeq Nano DNA kit, pooled in an equimolar fashion, and subjected to 2 250 paired-end sequencing on the Illumina MiSeq system. Sequencing reads were analyzed as described previously (22) but were demultiplexed based on library indices instead of internal bar codes. As before, background error hot spots (mostly G-to-T and C-to-A transversions) were identified using the plasmid controls and were masked prior to mutational analysis of the biological samples. The final sequencing data were also used in separate analyses that were focused on drug interactions between 5-aza-C and RNR inhibitors (J. M. O. Rawson, M. E. Roth, J. Xie, M. B. Daly, C. L. Clouser, S. R. Landman, C. S. Reilly, L. Bonnac, B. Kim, S. E. Patterson, L. M. Mansky, submitted for publication). Determination of 5-aza-CTP and 5-aza-dCTP levels by LC–MS-MS. Samples were prepared for liquid chromatography-tandem mass spectrometry (LC–MS-MS) by splitting U373-MAGI cells onto 10-cm plates (1.3 million cells/plate), using two plates per treatment group. The medium was

replaced 24 h later, and 5-aza-C, 5-aza-dC, or DMSO (for the no-drug control) was added. 5-Aza-C and 5-aza-dC were added at equivalent effective concentrations (i.e., EC25, EC50, or EC75). Cells were collected 4 h after 5-aza-C or 5-aza-dC addition, corresponding to the time of early reverse transcription (data not shown). Cell pellets were then resuspended in 750 ul of 60% methanol (stored at 20°C) and were incubated at 20°C for 18 h. The samples were vortexed, heated at 95°C for 3 min, and centrifuged at 16,000 g for 5 min. The supernatants were transferred to new microcentrifuge tubes, dried using a Savant SPD1010 SpeedVac concentrator (Thermo Fisher Scientific, Inc.) at maximum pressure, and frozen at 80°C until the time of analysis. For LC–MS-MS, dried extracts were dissolved in 200 ul of water containing 10 uM 5-iodo-dCTP, an internal standard. The samples were centrifuged at 14,000 rpm for 5 min at 4°C, and the supernatants were analyzed by LC–MS-MS. 5-Aza-C and 5-aza-dC samples were reconstituted immediately prior to LC–MS-MS injection in order to minimize their degradation during the procedure. LC–MS-MS was used to determine the levels of 5-aza-CTP, 5-aza-dCTP, riboguanylurea 5=-triphosphate (RGU-TP; the final hydrolysis product of 5-aza-CTP), and 2=-deoxyriboguanylurea 5=-triphosphate (dRGU-TP; the final hydrolysis product of 5-aza-dCTP) by a method published previously (23) with minor modifications. Purified 5-aza-CTP and 5-aza-dCTP were included as standards to verify the procedure. The LC–MS-MS system consisted of an AB Sciex QTrap 5500 mass spectrometer and an Agilent 1260 Infinity high-performance liquid chromatography (HPLC) system. Chromatographic separation of analytes was achieved using a Thermo Scientific Hypercarb column (length, 100 mm; inside diameter, 3 mm; particle size, 5 um). The two eluents were 0.5% diethylamine in water, with the pH adjusted to 10 with acetic acid (A), and 50% acetonitrile in water (B). The mobile phase was delivered at a flow rate of 0.5 ml/min using stepwise gradients of A and B as follows: from 0 to 20 min, 0 to 25% (vol/vol) B; from 20 to 28 min, 25 to 50% (vol/vol) B; from 28 to 28.5 min, 50 to 95% (vol/vol) B; from 28.5 to 30.5 min, 95% (vol/vol) B; from 30.5 to 31 min, 95 to 0% (vol/vol) B; from 31 to 39 min, 0% (vol/vol) B. Only the eluate from 10 to 30 min was diverted into the mass

spectrometer for analysis. The analytes were detected by MS-MS using an electrospray ionization (ESI) ion source with multiple reaction monitoring (MRM) detection in negative mode, with the curtain gas set to 20 lb/in2 . The ion spray voltage was set at 4,500 V and the temperature at 650°C. The nebulizer gas (GS1) and turbo gas (GS2) were both set at 45 lb/in2 . HIV-1 RT in vitro incorporation assay. The HIV-1 RT single nucleotide extension assay was performed as in a previous study (24) but with minor modifications. Briefly, a 32P 5=-end-labeled DNA primer of 18 nucleotides (nt) (5=-GTCCCTCTTCGGGCGCCA-3=) was annealed to a DNA template of 19 nt (3=-CAGGGAGAAGCCCGCGGTG-5=) at a 1:2 ratio. Single nucleotide extension indicates that the compound of interest (5-aza-CTP, 5-aza-dCTP, CTP, or dCTP) was successfully incorporated. The reaction mixtures contained 200 fmol template-primer, 2 ul of the test compound at the indicated concentrations (or 2 ul of dNTPs, for a final concentration of 50 uM for the positive control), 4 ul of purified RT (HIV-1 NL4-3), 25 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 100 mM KCl, 5 mM MgCl2, and 10 uM oligo(dT), for a final volume of 20 ul/ reaction mixture. Reaction mixtures were incubated at 37°C for 5 min, and the reactions were then quenched with 10 ul of 40 mM EDTA and 99% (vol/vol) formamide at 95°C for 2 min. The reactions were resolved on a 14% urea-PAGE gel (AmericanBio, Inc., Natick, MA), and the results were analyzed using a PharosFX molecular imager (Bio-Rad) and Image Lab software (Bio-Rad). Statistical analyses. All figures were created in Microsoft Office for Mac 2011, version 14.5.2 (Redmond, WA), or GraphPad Prism, version 5.0 (GraphPad Software, Inc., La Jolla, CA). To determine the EC25, EC50, and EC75 of 5-aza-C and 5-aza-dC, infectivity data were normalized to the data for the no-drug control, plotted against log-transformed drug concentrations, and subjected to nonlinear regression in GraphPad Prism. In order to determine whether differences in 5-aza-dCTP levels between cells treated with 5-aza-C and those treated with 5-aza-dC were statistically significant, normalized data were analyzed by one-way repeated-measures analysis of variance (ANOVA) (without assuming equal variability of differences) in GraphPad Prism. Sidak's posttest was used to compare the relative quantity of 5-aza-dCTP at

each effective concentration (i.e., EC25, EC50, or EC75). To test for variables that may influence mutation frequencies, generalized linear mixed-effects models were applied to processed Illumina sequencing data. The raw counts for each type of mutation (e.g., transitions) were modeled as overdispersed Poisson random variables, with an offset given by the total number of reference bases. The type of sample, the type of amplicon, and their interactions were treated as fixed effects, while the replicate was treated as a random effect. The logarithmic link was used, as is standard for Poisson outcomes, and penalized quasi-likelihood was used to estimate the model parameters (25). These computations were conducted using R, version 3.1.0, and the MASS package. Nucleotide sequence accession number. All Illumina sequencing data supporting the results of the manuscript have been deposited into the NCBI Sequence Read Archive (SRA) under accession no. SRP068916.

RESULTS

5-Azacytidine and 5-aza-2'-deoxycytidine induce similar levels of G-to-C and C-to-G transversions in HIV-1. In order to determine the primary form of 5-aza-C that is active against HIV-1, the effects of 5-aza-C and 5-aza-dC on HIV-1 mutagenesis were compared using Illumina amplicon sequencing. The direct incorporation of 5-aza-C as a ribonucleotide (5-aza-CTP) was predicted to lead to a mutational pattern distinct from that of 5-aza-dCTP, because incorporated ribonucleotides can (i) be replaced (correctly or incorrectly) by RNase H2-mediated repair, (ii) cause mutations if left unrepaired, particularly short (2- to 5-bp) deletions, and (iii) elevate the HIV-1 RT mismatch extension frequency on the opposite strand (26–28). To prepare samples for sequencing, U373-MAGI cells were treated with DMSO (i.e., no drug), 5-aza-C (EC75, 260 uM) or 5-aza-dC (EC75, 3.8 uM). Cells were then infected 2 h after drug addition at an MOI of 1.0 with NL4-3 MIG-VSVG. Genomic DNA was purified from cells collected 72 h postinfection, and PCR was performed to prepare multiple amplicons (Gag, Pol, Vif, Env, Nef) from proviral DNA. Plasmid control amplifications were included to measure the level of background error

from PCR and sequencing. The amplicons were pooled, used to prepare libraries, and subjected to 2 250 paired-end sequencing on the Illumina MiSeq system. Illumina sequencing generated 5.5 million read pairs after all bioinformatics processing steps, containing 570,000 mutations and 630 million reference bases. 5-Aza-C and 5-aza-dC were found to significantly increase the frequencies of G-to-C and C-to-G transversions compared to the no-drug control in all amplicons examined (P, 0.0001 for all comparisons), although C-to-G transversions were 5- to 6-fold less prevalent than G-to-C transversions (Fig. 1). In the presence of 5-aza-C, 67% of all mutations were G-to-C transversions and 12% were C-to-G transversions (compared with 64% and 12% for 5-aza-dC). C-to-G transversions were not observed previously when 5-aza-C was added during the early phase of viral replication (10), likely due to the lower sequencing depth of earlier studies (see Discussion). Notably, 5-aza-C and 5-aza-dC increased the levels of G-to-C transversions (Fig. 1A) and C-to-G transversions (Fig. 1B) to similar extents in all five amplicons, and the resulting mutation frequencies did not differ signifi- cantly (P, 0.05 for all comparisons). Depending on the amplicon, 5-aza-C and 5-aza-dC raised G-to-C transversion frequencies by 45- to 75-fold and C-to-G frequencies by 25- to 60-fold. Further, we did not observe short (2- to 5-bp) deletions that are characteristic of ribonucleoside incorporation in the 5-aza-C-treated sample (data not shown). These data demonstrate that 5-aza-C and 5-aza-dC cause very similar changes in viral mutation frequencies and spectra, arguing that 5-aza-C acts primarily as 5-aza-dCTP during reverse transcription.

5-Azacytidine and 5-aza-2’-deoxycytidine cause highly similar HIV-1 mutational patterns. 5-Aza-dC has been demonstrated previously to cause highly variable levels of G-to-C and C-to-G transversions depending on the specific sequence position (12). If 5-aza-C acts against HIV-1 after reduction to 5-aza-dC, the susceptibilities of individual bases to 5-aza-C and 5-aza-dC should closely resemble one another. To address this, mutation frequencies were determined at individual sequence positions in the five amplicons. G-to-C mutation frequencies were

determined at every guanine position (124 in total), while C-to-G mutation frequencies were determined at every cytosine position (116 in total). The extent of correlation between 5-aza-C- and 5-aza-dCinduced mutation frequencies was then examined. For both 5-aza-C and 5-aza-dC, significant variability (as much as 20- fold) was observed between G-to-C and C-to-G frequencies at individual sequence positions (Fig. 2). 5-Aza-C- and 5-aza-dCmediated mutation frequencies were found to be strongly positively correlated, both for G-to-C transversions (Pearson's r, 0.93 [95% confidence interval {CI}, 0.90 to 0.95]; P 0.0001) and for C-to-G transversions (Pearson's r, 0.96 [95% CI, 0.94 to 0.97]; P 0.0001). This correlation was able to explain most of the variability in the observed data (R2 , 0.87 [G-to-C transversions] or 0.91 [C-to-G transversions]). The slopes of the best-fit linear regression lines were also determined, since they would equal 1.0 if the 5-aza-C- and 5-aza-dC-induced mutation frequencies matched perfectly. The regression lines exhibited slopes of 0.71 for G-to-C transversions (95% CI, 0.66 to 0.76) and 0.88 for C-to-G transversions (95% CI, 0.83 to 0.93), indicating that the frequencies of 5-aza-C-mediated mutations were slightly higher than those of 5-aza-dC-mediated mutations. These findings indicate that the patterns of viral mutagenesis in the presence of 5-aza-C and 5-aza-dC closely resembled one another, further indicating that 5-aza-C likely reduces HIV-1 infectivity after conversion to 5-aza-dC. Levels of 5-aza-dCTP are similar in cells treated with 5-azacytidine or 5-aza-2=-deoxycytidine. While a previous report indicated that 10 to 20% of 5-aza-C is reduced to 5-aza-dC in one particular cell line (13), the conversion of 5-aza-C to 5-aza-dC has not yet been assessed in cell lines for which antiviral activity has been demonstrated. To address this, cells were treated with multiple concentrations of either 5-aza-C or 5-aza-dC for 4 h (corresponding to a time point during which reverse transcription should be actively occurring); nucleotides were then extracted, and LC–MS-MS was performed to determine the relative quantities of 5-aza-CTP and 5-aza-dCTP (see Materials and Methods). The relative levels of RGU-TP and dRGU-TP, which are the final hydrolysis products of 5-aza-CTP and 5-aza-dCTP, respectively, and may be relevant to antiviral activity, were also determined. Cells were treated

with equivalent effective concentrations (EC25, EC50, or EC75) of 5-aza-C or 5-aza-dC. If 5-aza-C acts primarily as 5-aza-dCTP against HIV-1, cells treated with 5-aza-C or 5-aza-dC should contain approximately equal levels of 5-aza-dCTP at equivalent effective concentrations. 5-Aza-CTP and RGU-TP were detected only in cells treated with 5-aza-C (data not shown), as expected considering the lack of cellular pathways for the conversion of deoxyribonucleotides to ribonucleotides. In contrast, 5-aza-dCTP was detected in cells treated with either 5-aza-C or 5-aza-dC (Fig. 3A), indicating that 5-aza-C (in its diphosphate form) was reduced to 5-aza-dC by RNR. Further, the levels of 5-aza-dCTP either did not differ significantly between cells treated with 5-aza-C and cells treated with 5-aza-dC (EC25 and EC75) or were higher in cells treated with 5-aza-C (at the EC50) (P 0.05). Similar trends were observed for dRGU-TP, the final hydrolysis product of 5-aza-dCTP (Fig. 3B). These findings further indicate that 5-aza-C reduces the infectivity of HIV-1 primarily after reduction to 5-aza-dC, since HIV-1 RT should incorporate 5-azadCTP much more readily than 5-aza-CTP. HIV-1 reverse transcriptase incorporates 5-aza-CTP in vitro much less efficiently than 5-aza-dCTP. Although similar levels of 5-aza-dCTP were observed in cells treated with 5-aza-C or 5-azadC, the extents to which 5-aza-CTP and 5-aza-dCTP are incorporated by HIV-1 RT had not yet been determined. 5-Aza-CTP was hypothesized to be incorporated only weakly, while 5-aza-dCTP was hypothesized to be incorporated relatively efficiently, since 5-aza-dCTP can be efficiently incorporated by cellular DNA polymerases (29, 30). To address this, the incorporation of 5-aza-CTP and that of 5-aza-dCTP were compared using an HIV-1 RT in vitro single nucleotide extension assay, with dCTP and CTP included for comparative purposes. Additional control reactions were performed by incubation with all four standard dNTPs (50 uM) (positive control) or by the omission of RT (negative control). It was found that HIV-1 RT incorporated 5-aza-dCTP relatively efficiently (Fig. 4), resulting in total primer extension at 5 uM (lane 10) and some detectable extension at 5-aza-dCTP concentrations as low as 50 nM (lane 8). However, 5-aza-dCTP was incorporated somewhat less effectively than dCTP (Fig. 4, compare lanes 7 to 9 with lanes 19 to 21). In contrast, HIV-1 RT incorporated 5-

aza-CTP and CTP only at the highest concentration tested (500 uM) (Fig. 4, lanes 6 and 18). These data demonstrate that 5-aza-CTP can be directly incorporated by HIV-1 RT, but only at very high concentrations and in the absence of competing deoxyribonucleotides. 5-Aza-dCTP was incorporated at least 10,000-fold more efficiently than 5-aza-CTP (Fig. 4, compare lanes 6 and 8). These observations are consistent with previous findings that HIV-1 RT exhibits high selectivity for deoxyribonucleotides, largely due to a steric gate (Y115) that blocks incoming ribonucleotides (14–16). Considering that similar levels of 5-aza-dCTP were observed in cells treated with equivalent effective concentrations of 5-aza-C or 5-aza-dC (Fig. 3), these findings strongly indicate that 5-aza-C exerts activity against HIV-1 primarily through its reduction to 5-aza-dC.

DISCUSSION

The ribonucleoside analog 5-aza-C could potentially act as a ribonucleotide or as a deoxyribonucleotide to reduce HIV-1 infectivity during the early phase of viral replication. To address this, Illumina sequencing was performed in order to compare the enhancement of HIV-1 mutagenesis by 5-aza-C and 5-aza-dC. 5-Aza-C and 5-aza-dC were found to cause similar changes in viral mutation frequencies regardless of the amplicon examined (Fig. 1). Both drugs elicited primarily G-to-C transversions in HIV-1, but also significant levels of C-to-G transversions, which were not observed in a previous study of 5-aza-C (10). 5-Aza-C was not found to induce other types of mutations that are considered indicative of ribonucleotide incorporation, such as short (2- to 5-bp) deletions (27). While the mechanism of C-to-G transversions remains unclear, they may result from the incorporation of 5-aza-dCTP into plus-strand viral DNA or, alternatively, from the direct incorporation of 5-aza-dCTP hydrolysis products (e.g., dRGU-TP) into minus-strand viral DNA. 5-Aza-C-mediated Cto-G transversions were not observed in a previous study (10), possibly due to the reduced depth of Sanger sequencing, the lower concentration of 5-aza-C used, or other differences in the mutational assay.

Additionally, 5-aza-C- and 5-aza-dC-mediated mutational patterns correlated strongly with one another even at the level of individual sequence positions in HIV-1 (Fig. 2), further indicating that 5-aza-C exerts antiviral activity after reduction to 5-aza-dC. 5-Aza-dCTP was detected in cells treated with 5-aza-C, indicating that 5-aza-C was reduced to 5-aza-dC (Fig. 3), and, importantly, similar levels of 5-aza-dCTP were observed in cells treated with equivalent effective concentrations of 5-aza-C and 5-aza-dC. Notably, the absolute concentrations of 5-aza-C were 70- to 300-fold higher than those of 5-aza-dC in these experiments, indicating that intracellular conversion of 5-aza-C to 5-aza-dCTP was much less efficient than the conversion of 5-aza-dC to 5-azadCTP. By use of an in vitro incorporation assay, very high concentrations of 5-aza-CTP were found to be successfully incorporated by HIV-1 RT (Fig. 4). However, 5-aza-dCTP was incorporated at least 10,000-fold more efficiently than 5-aza-CTP, further supporting the notion that 5-aza-dCTP is the principal form of 5-aza-C that is active against HIV-1. Taken together, these findings clearly indicate that 5-aza-dCTP is the primary form of 5-aza-C that is active during the early phase of HIV-1 replication. Further, 5-aza-C has been shown to exert much more potent antiviral activity during the early phase of replication than during the late phase of replication (10), indicating that 5-aza-C likely acts primarily as 5-aza-dCTP during spreading infections as well. These observations lead to the following model for the antiviral activities of 5-aza-C and 5-aza-dC (Fig. 5). 5-Aza-C and 5-aza-dC enter the cell by facilitated diffusion through a transporter protein and are then phosphorylated by the appropriate cellular kinases. 5-Aza-CTP is incorporated into viral RNA during the late phase of replication, leading to the fixation of C-to-G transversions during the following round of reverse transcription. 5-Aza-C (in its diphosphate form) is also converted to 5-aza-dC by RNR, and the resulting 5-aza-CDP can then be phosphorylated to form 5-aza-dCTP. Lastly, 5-aza-dCTP is incorporated into viral DNA during reverse transcription, resulting in the fixation of G-to-C and (to a lesser extent) C-to-G transversions in HIV-1. In the future, it would be of interest to investigate the antiviral activity of 5-aza-C in other cell types, such as primary CD4 T cells and macrophages. 5-Aza-C would likely

act primarily as 5-aza-dCTP in activated CD4 T cells, since they divide rapidly and thus have high levels of RNR and dNTPs (17, 31). In contrast, 5-aza-CTP could potentially be directly incorporated at significant levels in macrophages, which have low levels of RNR, low dNTP pools, and much higher ratios of NTPs to dNTPs (17, 31). Indeed, previous work has shown that HIV-1 RT can incorporate significant levels of endogenous ribonucleotides in macrophages and in vitro under macrophagelike conditions (17, 18). Further, the ribonucleoside analog 3=-deoxyadenosine, which likely cannot be reduced, exhibits antiviral activity in macrophages but not in CD4 T cells, indicating that ribonucleoside analog triphosphates can be directly incorporated in macrophages (17). Notably, the findings in this study have important implications for the design of ribonucleoside analogs directed against retroviruses. Ribonucleoside analogs are of interest as antiretroviral agents because their synthesis is often less complicated, less time-intensive, and more affordable than that of their de oxyribonucleoside counterparts. Ribonucleoside analogs may also offer other advantages in certain instances, such as better activation by cellular enzymes or improved antiviral activity by the targeting of both viral RNA and DNA. However, the findings in this study suggest that it is important to consider the efficiency at which ribonucleoside analogs are reduced—at least for the targeting of viral replication in cell types with high dNTP pools, such as CD4 T cells. In contrast, efficient reduction may not be necessary or even desired for the targeting of viral replication in cell types with low dNTP pools, such as macrophages. Ribonucleoside analogs that cannot be reduced (such as those lacking a 3= hydroxyl group, e.g., 3=-deoxyadenosine) could be used to specifically target viral replication in macrophages, an important viral reservoir (17). In sum, these findings inform efforts to identify additional nucleoside analogs that target HIV-1 RT, which continue due to significant concerns regarding the development of drug resistance and off-target effects.

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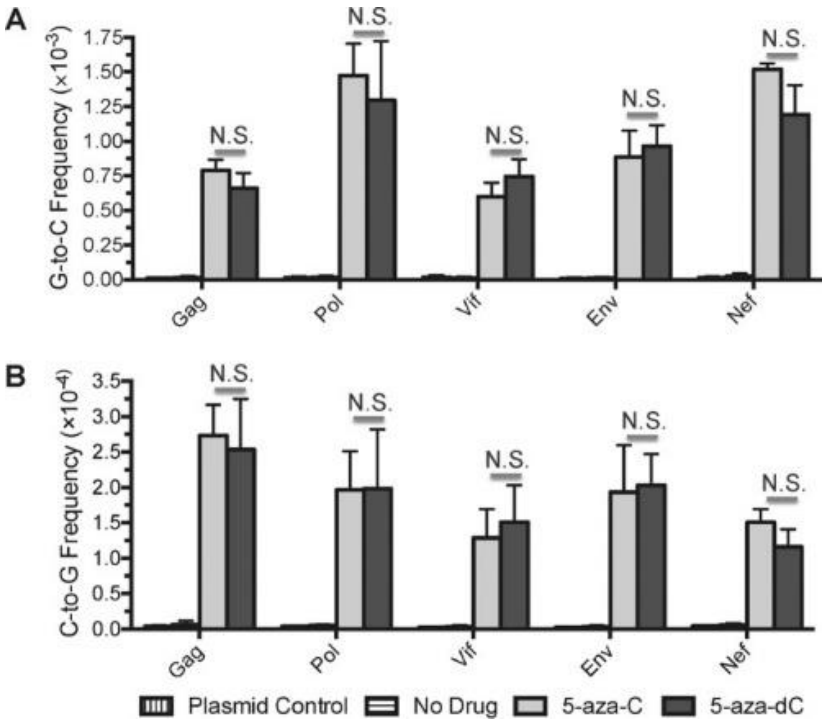

Figure 1: 5-Azacytidine and 5-aza-2′-deoxycytidine induce similar levels of G-to-C and C-to-G transversion mutations during HIV-1 replication. In order to determine whether 5-azacytidine (5-aza-C) and 5-aza-2′-deoxycytidine (5-aza-dC) induce similar changes in HIV-1 mutation frequencies and spectra, U373-MAGI cells were treated with DMSO (no-drug control), 5-aza-C, or 5-aza-dC. 5-Aza-C and 5-aza-dC were added 2 h before infection at the EC_{75} (~260 or 3.8 μM, respectively). Cells were infected at an MOI of 1.0 with NL4-3 MIG-VSVG and were collected 72 h postinfection for the purification of genomic DNA. PCR was performed to prepare multiple amplicons (Gag, Pol, Vif, Env, Nef) from proviral DNA; these were then pooled, used to prepare libraries, and analyzed by 2 × 250 paired-end sequencing on the Illumina MiSeq system. Plasmid control amplifications were performed to determine the levels of background errors resulting from PCR and sequencing. Mutation frequencies for each amplicon, expressed as the number of mutations per base pair, were calculated by dividing the number of mutations by the number of reference bases (mutations + wild-type bases). Data represent means ± standard deviations for three independent biological replicates; N.S., not significant ($P > 0.05$)

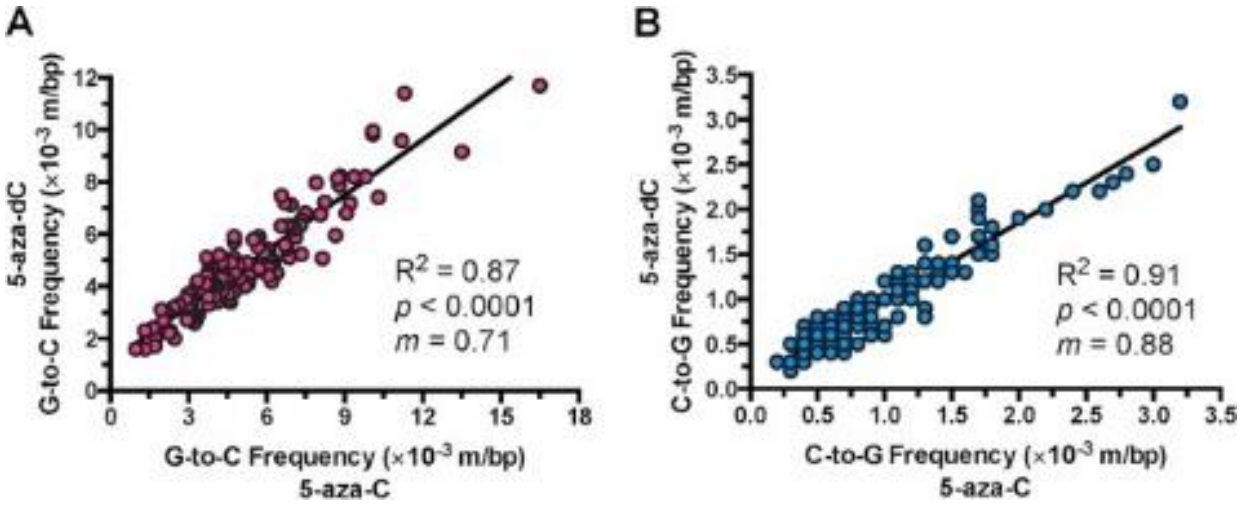

Figure 2: 5-Azacytidine and 5-aza-2′-deoxycytidine induce similar patterns of mutation during HIV-1 replication. Using the Illumina sequencing data, G-to-C and C-to-G transversion frequencies were determined at every individual guanine (124 in total) or cytosine (116 in total) position within the sequences of the five amplicons. Mutation frequencies for each amplicon were calculated by dividing the number of mutations by the number of reference bases (mutations + wild-type bases) and are represented as mutations per base pair (m/bp). 5-Aza-C- and 5-aza-dC-induced mutation frequencies were then plotted against each other for each sequence position, and the resulting data were subjected to linear regression and correlation analyses. Data represent averages for three independent biological replicates. R^2 denotes the extent to which the best-fit regression line explains the observed variability in the data; *P* indicates the significance of the correlation; and *m* indicates the slope of the best-fit regression line.

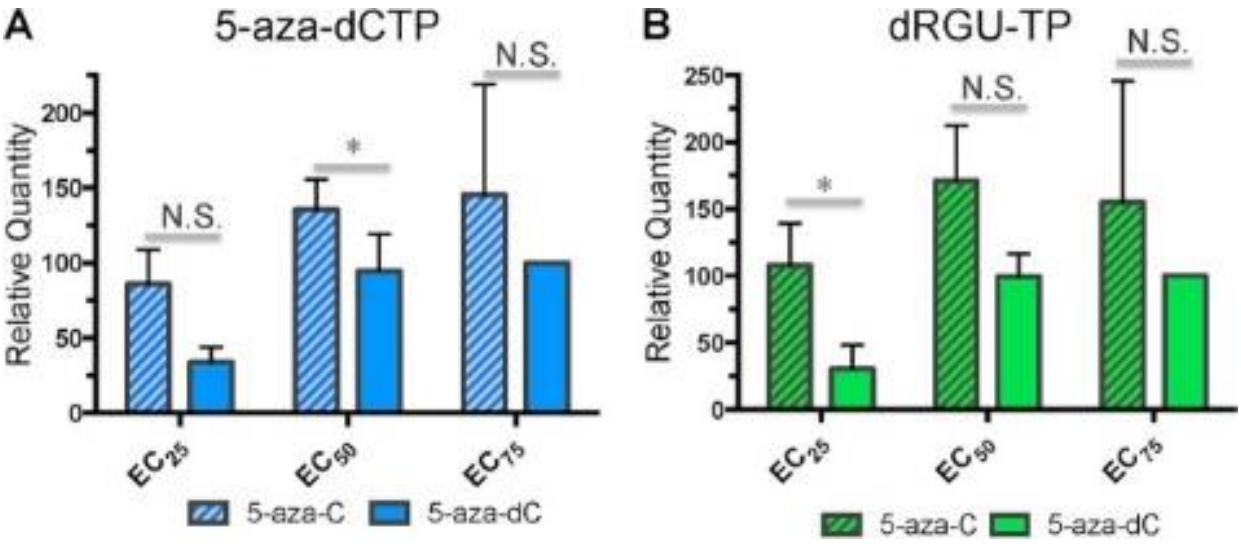

Figure 3: 5-Aza-dCTP levels are comparable in cells treated with 5-aza-C or 5-aza-dC. In order to determine the extent to which 5-aza-C is reduced to 5-aza-dC intracellularly, U373-MAGI cells were incubated with varying concentrations (EC_{25}, EC_{50}, or EC_{75}) of 5-aza-C or 5-aza-dC. Cells were collected for analysis 4 h after drug addition, a time corresponding to the expected time of early reverse transcription. LC–MS-MS was then used to determine the relative levels of 5-aza-dCTP and 2′-deoxyriboguanylurea 5′-triphosphate (dRGU-TP). dRGU-TP is the final hydrolysis product of 5-aza-dCTP and is potentially relevant to antiviral activity. Data represent means ± standard deviations for three independent experiments, normalized to the EC_{75} of 5-aza-dC. N.S., not significant; *, $P < 0.05$.

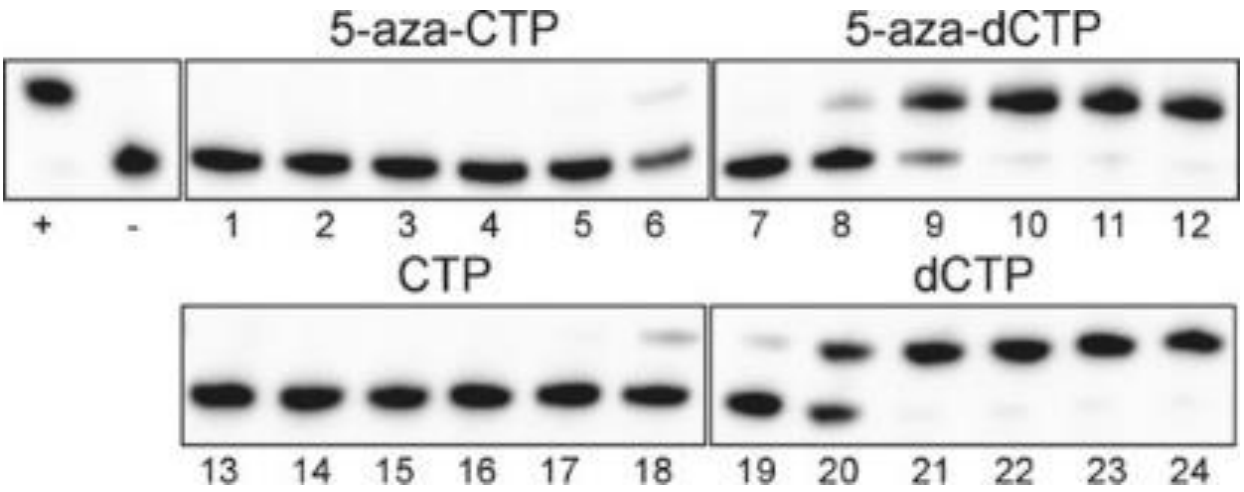

Figure 4: HIV-1 RT incorporates 5-aza-CTP much less efficiently than 5-aza-dCTP *in vitro*. The relative abilities of HIV-1 RT to incorporate 5-aza-CTP, 5-aza-dCTP, CTP, and dCTP were determined using an *in vitro* single nucleotide extension assay. The HIV-1 RT was incubated with a radiolabeled primer (18 nt) annealed to a DNA template (19 nt) in the presence of each compound at 5 nM to 500 μM (a 10-fold series, from left to right). Additional control reactions were performed by incubating with all four standard dNTPs (50 μM) (+) or by omitting RT (−). All of the reactions were analyzed on the same gel.

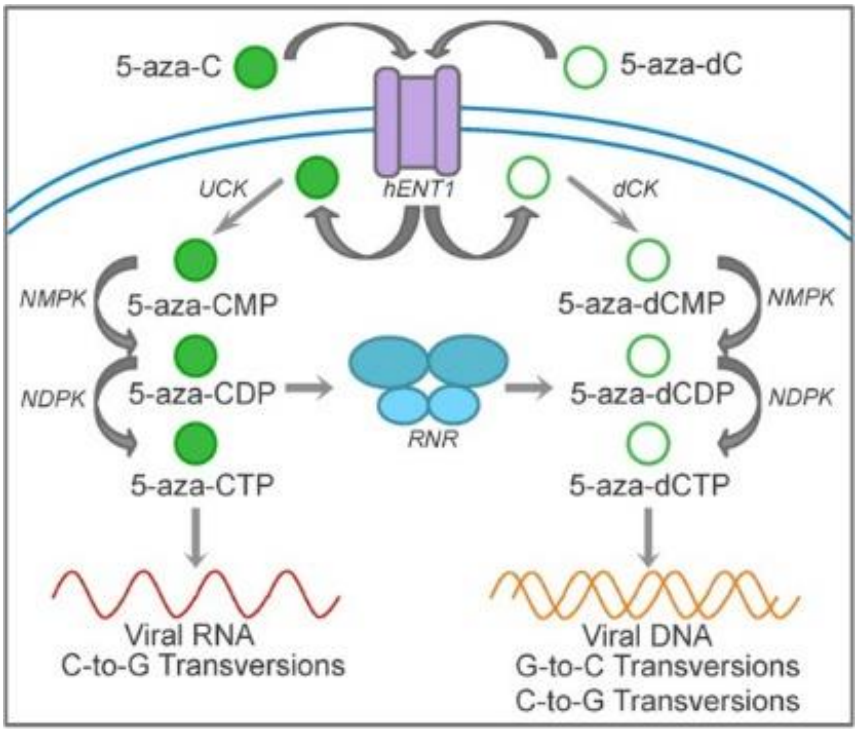

Figure 5: Model of 5-azacytidine- and 5-aza-2′-deoxycytidine-mediated HIV-1 mutagenesis. 5-Aza-C and 5-aza-dC are first transported into the cell by facilitated diffusion through human equilibrative nucleoside transporter 1 (hENT1). 5-Aza-C and 5-aza-dC are then phosphorylated by uridine-cytidine kinase (UCK) and deoxycytidine kinase (dCK), respectively, to the monophosphate forms. Nucleoside monophosphate and diphosphate kinases (NMPK and NDPK) phosphorylate the monophosphate and diphosphate forms, respectively, resulting in the formation of 5-aza-CTP and 5-aza-dCTP. 5-Aza-CTP can be incorporated during the transcription of viral genomic RNA, resulting in C-to-G transversions. 5-Aza-CDP is also reduced to 5-aza-dCDP, ultimately forming 5-aza-dCTP. 5-Aza-dCTP is incorporated during reverse transcription, resulting primarily in G-to-C transversions (reflecting minus-strand incorporation) but also in low levels of C-to-G transversions. The mechanism by which C-to-G transversions are formed is still unclear, but they may be due to plus-strand incorporation of 5-aza-dCTP or minus-strand incorporation of 5-aza-dCTP hydrolysis products.

Chapter IV: Anti-HIV Host Factor SAMHD1 Regulates Viral Sensitivity to Nucleoside Reverse Transcriptase Inhibitors via Modulation of Cellular dNTP Levels

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Contribution:

M.Daly contributed the data for Figure 4 (part C,D,E and F). The data generated in Figure 4 (part A and B) and Supplemental Figure 1 was done in conjunction with S. Amie.

Abstract

Newly identified anti-HIV host factor, SAMHD1, restricts replication of lentiviruses such as HIV-1, HIV-2, and SIV in macrophages by enzymatically hydrolyzing and depleting cellular dNTPs, which are the substrates of viral DNA polymerases. HIV-2 and some SIVs express Vpx, which counteracts SAMHD1 and elevates cellular dNTPs, enhancing viral replication in macrophages. Since nucleoside reverse transcriptase inhibitors (NRTIs), the most commonly used anti-HIV drugs, compete against cellular dNTPs for incorporation into proviral DNA, we tested whether SAMHD1 directly affects the efficacy of NRTIs in inhibiting HIV-1. We found that reduction of SAMHD1 levels with the use of virus-like particles (VLPs) expressing Vpx and SAMHD1 specific shRNA, subsequently elevates cellular dNTPs and significantly decreases HIV-1 sensitivity to various NRTIs in macrophages. However, VLP +Vpx treatment of activated CD4+ T cells only minimally reduced NRTI efficacy. Furthermore, our HPLC-based assay could not detect SAMHD1-mediated hydrolysis of various NRTI-triphosphates tested in this study, suggesting that the reduced sensitivity of HIV-1 to NRTIs upon SAMHD1 degradation is most likely caused by the elevation of the cellular dNTP levels.

Background

Human immunodeficiency virus types 1 (HIV-1) and 2 (HIV-2) and other lentiviruses replicate in CD4+ T cells and non-dividing, terminally differentiated macrophages during the course of viral pathogenesis (1,2). Unlike CD4+ T cells, which die upon HIV-1 infection (3), macrophages are resistant to the cytopathic effects of the virus and serve as key long-living viral reservoirs that persistently produce virus at a slow rate (2,4-8). Also, macrophages are found at sites of primary viral transmission and in many tissues and organs such as the brain, making infected macrophages an essential cell type to target therapeutically (9). Another fundamental difference between T cells and macrophages is that T cells are rapidly dividing while

macrophages remain in the G0 phase of the cell cycle and do not require chromosomal DNA replication. As a consequence, macrophages contain very limited cellular dNTP pools (22-320-fold lower) compared to activated peripheral blood mononuclear cells (PBMCs), primarily composed of T cells (10,11). In addition to the lack of cell cycling, it has recently been reported that expression of sterile alpha motif (SAM) and HD domain-containing protein 1 (SAMHD1) also contributes to the low dNTP pools found not only in macrophages, but other non-dividing cell types such as resting CD4+ T cells and dendritic cells (12-15). SAMHD1 acts by hydrolyzing deoxynucleoside triphosphates (dNTPs) into deoxynucleosides (dNs) and triphosphates (16,17) and acts as an anti-viral host defense factor to restrict HIV-1 infection in these cell types by depleting the dNTP substrate availability for HIV-1 reverse transcriptase (RT) (12-15,18). However, HIV-2 and several simian immunodeficiency viruses (SIVs) counteract SAMHD1 restriction by expression of an accessory protein, Vpx, which is able to target SAMHD1 for proteasomal degradation (13,19-24). Degradation of SAMHD1 results in an increase in cellular dNTPs, an acceleration of proviral DNA synthesis, and ultimately enhanced viral replication in non-dividing cells such as macrophages (12,15,18).

Nucleoside reverse transcriptase inhibitors (NRTIs) are the most commonly used agents in highly active anti-retroviral therapy (HAART), which significantly delays HIV-1 pathogenesis (25,26). NRTIs lack the 3' OH moiety of the ribose ring and are typically administered as their nucleoside derivatives to facilitate crossing of cellular membranes. Once inside the cell, NRTIs are phosphorylated by host nucleoside and nucleotide kinases to their triphosphate form (NRTI-TP), which competes with cellular dNTPs as substrate for viral RT. Incorporation of NRTI-TPs into HIV-1 proviral DNA halts DNA chain elongation by preventing the formation of the next phosphodiester bond with an incoming dNTP (reviewed in, (27)).

Since all NRTIs used for the treatment of HIV-1 and HIV-2 infected patients compete against cellular dNTPs, we hypothesized that SAMHD1 activity alters the efficacy of NRTIs in

inhibiting viral replication. One mechanism by which SAMHD1 may influence NRTI efficacy toward HIV-1 is direct hydrolysis of NRTI-TPs to their inactive form, thus decreasing the active concentration of the drug in the cell. The other possible mechanism would occur upon degradation of SAMHD1, which results in an increase in cellular dNTPs (12) and would likely decrease the efficacy of NRTIs by providing more competitive dNTP substrate for RT. This would likely influence NRTI efficacy toward HIV-2. Indeed, our virological and biochemical results demonstrate that the reduction of SAMHD1 expression almost abolishes viral sensitivity to NRTIs in macrophages by elevating cellular dNTPs. This suggests that higher concentrations of NRTIs may be necessary to inhibit HIV-2 infection in macrophages. Furthermore, we could not detect SAMHD1-mediated hydrolysis of NRTI-TPs by our HPLC-based assay suggesting that SAMHD1 does not significantly alter the active concentration of the drug. Overall, our data suggest that the anti-HIV host factor SAMHD1 directly enhances the efficacy of NRTIs in inhibiting HIV-1 infection in macrophages by selectively hydrolyzing dNTPs much more efficiently than NRTI-TPs, thus increasing the likelihood that RT incorporates these DNA chain terminators.

The abbreviations used are: HIV-1, human immunodeficiency virus type 1; HIV-2, human immunodeficiency virus type 2; SIV, simian immunodeficiency virus; RT, reverse transcriptase; NRTIs, nucleoside reverse transcriptase inhibitors; NRTI-TPs, NRTI triphosphates; NNRTIs, non-nucleoside reverse transcriptase inhibitors; SAMHD1, sterile alpha motif and HD domain containing protein 1; VLPs, virus-like particles; HAART, highly active antiretroviral therapy; AZT, zidovudine; ddC, zalcitabine; ABC, abacavir; TDF, tenofovir; ddNTPs, dideoxynucleoside triphosphates; hpt, hours post transduction; dpt, days post transduction; HIV-1 D3, HIV-1 NL4-3, VSVG pseudotyped vector; NDK, nucleoside diphosphate kinase.

Experimental Procedures

Ethics Statement - Experiments in this study utilized primary human monocytes and CD4+ T cells isolated from human buffy coats (New York Blood Services, Long Island, NY). Human blood is publically available for purchase, and is provided without indicating the identity of the subjects.

Cell Cultures - THP1 cell lines expressing a scrambled shRNA (5'-CCGGCAACAAGATGAAGAGCACCAACTC GAGTTGGTGCTCTTCATCTTGTTGTTTTT-3') or a SAMHD1 specific shRNA (5'- CCGGGCAGATGACTACATAGAGATTCTC GAGAATCTCTATGTAGTCATCTGCTTTTT TG-3', provided by Dr. Nathaniel Landau) were prepared as previously described (28). Cells were maintained in RPMI (10% FBS with penicillin and streptomycin) with 0.5 µg/ml puromycin for selection. THP1 cells were differentiated overnight with 50 nM phorbol-12- myristate-13 acetate (PMA, Sigma Aldrich). Primary human monocytes were isolated with MACS CD14 beads (Miltenyi Biotec) and CD4+ T cells were isolated with CD4 beads (Miltenyi Biotec) from human blood buffy coats as described previously (29). Monocytes were differentiated to macrophages with 5 ng/ml human GM-CSF (Miltenyi Biotec) in RPMI for 7 days. CD4+ T cells were activated for 5 days in the presence of 20 U/ml IL2 (Miltenyi Biotec) with 5 µg/ml phytohemagglutinin (PHA, Sigma Aldrich) added only on day 1.

*HIV-1 D3 vector and VLP preparatio*n - The HIV-1 D3 plasmid encodes the genome for HIV-1 NL4-3 with eGFP in the place of nef and a deleted envelope (10). Vector and virus-like particles (VLPs) were prepared by the protocols previously described .

Purification of RT and SAMHD1 - HXB2 HIV-1 RT (p66) gene was cloned into pET28a (Novagen), containing an N-terminal hexahistidine-tag. RT homodimer was expressed in BL21 (DE3) E. coli and purified using three forms of chromatography: Ni2+- nitrilotriacetic acid (NTA), diethylaminoethanol Sepharose (DEAE), and sulfopropyl (SP) anion exchange (30).

Human SAMHD1 was cloned into pGEX-6P-1 with an N-terminal GST tag (GE Healthcare, provided by Dr. Yoshio Koyanagi) and transformed into BL21 (DE3) pLysS competent cells (Invitrogen). Cells were grown at 37° C to an OD600 of 0.5, stored on ice for 2 h, and induced overnight with 0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 25° C. Cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 2 mM EDTA, 1 mg/ml chicken egg white lysozyme, 1 tablet Roche Complete protease inhibitor cocktail) for 4 h on ice. Cell debris was removed by centrifugation at 49,000 x g for 15 min and lysate was incubated overnight at 4°C with 1.5 ml glutathione Sepharose 4B bead slurry (GE Healthcare). Beads were pelleted and washed 3 times in wash buffer (50 mM TrisHCl pH 7.5, 500 mM NaCl, 1 mM dithiothreitol, 0.5% Triton-X 100), equilibrated in buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 20% glycerol, 0.5% Triton-X 100) and packed into a column. The column was washed 2 times with 10 ml equilibration buffer and SAMHD1 was eluted with 50 mM Tris-HCl pH 8, 1 mM EDTA, 10% glycerol, 300 mM NaCl, 300 mM reduced glutathione. Eluted fractions were separated on 10% SDS-PAGE and stained with Coomassie Blue to determine protein concentration by comparing to a bovine serum albumin (BSA) standard curve. Protein was dialyzed overnight in equilibration buffer.

Western blot analysis - THP1 cells were differentiated overnight in PMA. Macrophages were treated with VLPs (150 ng p27 per 1 million cells) for 24 h and activated CD4+ T cells were treated with VLPs (375 ng p27 per 1 million cells) for 24 h. To ensure VLPs entered the T cells, cells were spinoculated at 1,670 x g for 5 min, resuspended, and spinoculated once more. All cells were washed in PBS, and lysed in RIPA buffer (10X PBS, 5% sodium deoxycholate, 10% SDS, nonyl phenoxypolyethoxylethanol (NP-40), Roche Complete protease inhibitor cocktail). 25 µg total protein from THP1 cells and macrophages and 50 µg total protein from CD4+ T cells were separated by 10% SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane (Bio-Rad) and SAMHD1 along with β-actin (loading control) were detected after incubation with

enzyme-specific antibodies (Abcam (1:1,000) and Sigma Aldrich (1:3,000), respectively) followed by incubation with an anti-mouse-HRP secondary antibody (GE Healthcare). Immunoreactive protein bands were detected by chemiluminescence (Thermo Scientific SuperSignal West Femto Maximum Sensitivity Substrate) and imaged on a Bio-Rad ChemiDoc Imager. SAMHD1 expression levels were quantified by densitometry (Quantity One) and normalized to the β-actin controls.

HIV-1 RT primer extension assay - Reactions (20 µl final) contained 50 nM purified RT, 10 nM 5' 32P-labeled 17-nt DNA primer (5'- CGCGCCGAATTCCCGCT-3') annealed to a 38-nt RNA template (3'- GCGCGGCUUAAGGGCGAUCGUUAUAAG ACGUCGGUUCG-5', Integrated DNA Technologies), increasing concentrations of dNTPs (10, 25, 50, 100, 250, 500 nM, 1 µM, USB Products), with or without 5 µM AZT-TP (Moravek Biochemicals), 5 µM ddCTP (SigmaAldrich), and RT reaction buffer (50 mM TrisHCl pH 7.5, 50 mM NaCl, 5 mM MgCl2, and 10 µM oligo-dT). Reactions were initiated by addition of RT, incubated for 10 min at 37° C, terminated by addition of 10 µl 40 mM EDTA, 99% formamide, and denatured by incubation for 5 min at 95° C. Denatured products (4 µl) were then separated by 16% urea PAGE (SequaGel, National Diagnostics) and imaged on a Bio-Rad Personal Molecular Imager.

Drug sensitivity assays - After differentiation (THP1 cells and monocytes), activation (T cells), and VLP pretreatment (macrophages and T cells) as described previously, NRTIs or nevirapine (National Institutes of Health AIDS Reagent Program) were added to the cells at the indicated concentrations. After pretreatment with NRTIs for 2 h to allow for phosphorylation, HIV-1 D3 was added. THP1 cells were collected for flow cytometry 48 hours post transduction (hpt), macrophages were collected 7 days post transduction (dpt) (media and drugs were replaced 48 hpt), and T cells were collected 40 hpt. Cells were stained with propidium iodide to monitor cell death, and the percentage of live/transduced (PI-/GFP+) cells were measured using an Accuri C6 Flow Cytometer, analyzed in FlowJo, and normalized to the no drug controls.

dNTP quantification assay - Activated CD4+ T cells were treated with VLPs +/-Vpx (375 ng p27 per 1 million cells) for 24 h and dNTPs were extracted and quantified using the protocol described by Diamond et al. (10). To obtain the concentration of dNTPs per cell, the volume of an activated CD4+ T cell, 320 µm3 (10), was used in the calculation.

SAMHD1 phosphohydrolysis assay - Reactions (35 µl final) contained 1 µM purified SAMHD1, 1 mM dNTP/ddNTP/AZT-TP, +/- 100 µM dGTP, and reaction buffer (50 mM Tris-HCl pH 8, 50 mM KCl, 5 mM MgCl2, and 0.1% Triton X-100). Reactions were initiated with the addition of SAMHD1, incubated for 3 h at 37° C, and terminated by incubation for 10 min at 75° C. Reactions were diluted in 12.5% acetonitrile containing 60 µM dCMP as a reference control and then injected into a Beckman Coulter System Gold 126 Solvent Module as described by White et al. (17,31). Nucleotide abundance was determined by integrating the area under each peak using 32 Karat 8.0 Software. dNTP/ddNTP/AZT-TP levels were normalized by the amount of dCMP detected in each diluted sample and then normalized to the no-enzyme controls. Reactions conducted to test allosteric activation with ddGTP were carried out as described above, but contained 500 µM of the indicated activator and were incubated for 30 min at 37° C.

Statistical Methods - Statistical significance was determined using an unpaired ttest with a Welch's correction. All error bars represent the standard error of the mean (SEM).

Results

Effect of SAMHD1 on HIV-1 sensitivity to NRTIs in THP-1 cells - Since SAMHD1 modulates the concentration of cellular dNTPs, which compete against NRTI-TPs as substrates for RT we hypothesized that SAMHD1 influences HIV-1 sensitivity to NRTIs. To test this, we first utilized a THP-1 cell line, which is a human monocytic cell line that endogenously expresses SAMHD1 (12,32). To knockdown SAMHD1, a SAMHD1-specific shRNA (shSAMHD1) was stably expressed in these cells, and a scrambled shRNA (shScramble) was used as a control.

Reduced SAMHD1expression (Figure 1A, (12,20)) and elevated dNTP levels in the shSAMHD1 cells after differentiation with PMA were observed (12). The differentiated shScramble and shSAMHD1 THP-1 cell lines were pretreated with increasing concentrations of zidovudine (AZT) and then transduced with a Vesicular Stomatitis Virus-G protein (VSV-g) pseudotyped HIV-1 D3 vector, which encodes the entire NL4-3 HIV-1 genome except env, and nef is replaced with eGFP (HIV- 1 D3, (10)). The transduction efficiency at each AZT concentration was measured by flow cytometry for eGFP expression 48 hpt, and inhibition curves were plotted to compare relative drug efficacy. Transduction efficiencies were normalized to the no drug controls since shSAMHD1 cells are more readily transduced than shScramble cells. As shown in Figure 1B, there was a 12.7-fold reduction in HIV-1 sensitivity to AZT at the highest concentration tested (200 nM) in the shSAMHD1 cells compared to the shScramble cells. This result shows that knockdown of SAMHD1 decreases HIV-1 sensitivity to NRTIs in THP-1 cells. However, when this experiment was repeated with a non-nucleoside RT inhibitor (NNRTI), nevirapine, the efficacy of nevirapine was not altered upon knockdown of SAMHD1 (Figure 1C). This result was predicted as NNRTIs bind to an allosteric site of HIV-1 RT and therefore, unlike NRTIs, do not compete against cellular dNTPs. These inhibition curves demonstrate that SAMHD1 specifically affects the efficacy of AZT, and not nevirapine, against HIV-1 replication.

Effect of Vpx on HIV-1 sensitivity to NRTIs in macrophages - Next, our hypothesis was tested in primary human monocyte-derived macrophages. To modulate the SAMHD1 level, we treated macrophages with VLPs containing or lacking Vpx (VLPs +/-Vpx) (20). As shown in Figure 2A, the SAMHD1 expression level in macrophages was reduced on average 6-fold upon treatment with VLPs +Vpx, and we previously reported that Vpx elevates cellular dNTP levels up to 33-fold in macrophages (12,18). After macrophages were pretreated with VLPs for 24 h, the cells were treated with NRTIs, AZT (Figure 2B), abacavir (ABC, Figure 2C), zalcitabine (ddC, Figure 2D), or tenofovir (TDF, Figure 2E), and then transducedwith HIV-1 D3. Cells were

collected for flow cytometry analysis 7 dpt. As shown in Figure 2B-E, HIV-1 transduced macrophages pretreated with VLPs +Vpx displayed significantly reduced sensitivity to the NRTIs tested, compared to cells treated with VLPs -Vpx. Furthermore, this reduction in drug efficacy could also be influenced by the level of NRTIs that become phosphorylated upon degradation of SAMHD1. It is possible that less NRTIs are phosphorylated by host kinases when the dNTP concentrations in the cell increase. Lahouassa et al. have demonstrated that when macrophages are treated with VLPs +Vpx for 24 h prior to dN treatment the concentration of dNTPs are 65-1,200-fold higher than when treated with VLPs +Vpx alone (12). This suggests that the kinases expressed in macrophages are active enough to phosphorylate a high concentration of dNs into dNTPs. However, nucleoside diphosphate kinase (NDK) is not as active toward NRTIs as canonical dNTPs (33). Therefore, when the concentration of dNTPs increases, the dNTPs may out compete NRTIs for the active site of NDK resulting in a decreased concentration of NRTITPs. Consequently, the reduced efficacy of NRTIs observed upon Vpx-mediated degradation of SAMHD1 in macrophages may be the result of direct competition of dNTPs for the active site of RT and for the active site of cellular kinases.

Effect of Vpx on HIV-1 sensitivity to multiple NRTIs in macrophages - In HAART, more than one NRTI is commonly used for the treatment of infected patients (34). Therefore, we tested whether Vpx-mediated SAMHD1 degradation is also capable of diminishing the anti-viral efficacy of multiple NRTIs when simultaneously applied to cells. We first biochemically tested how well combination treatment with both zidovudine-triphosphate (AZT-TP) and zalcitabine-triphosphate (ddCTP) inhibited RT primer extension at increasing dNTP concentrations (Figure 3A). In this assay, purified HIV-1 RT protein was incubated with a 5' 32P–labeled 17-nt DNA primer (“P” in Figure 3A) annealed to a 38-nt RNA template with increasing dNTP concentrations and a fixed concentration of AZT-TP and ddCTP. As shown in Figure 3A, HIV-1 RT was able to fully extend the primer (“F”) at all dNTP concentrations used in the absence of

drugs, but when 5 μM AZT-TP and ddCTP were added to the reactions, extension with 10-50 nM dNTPs (equivalent to dNTP levels in macrophages expressing SAMHD1, see "M") was significantly reduced. Inhibition under these reaction conditions is indicated by reduced levels of the full-length products and the presence of early terminated products with variable sizes (marked with asterisks) that are not present in the no NRTI-TP controls. However, at the approximate dNTP concentrations found in activated CD4+ T cells (1-16 μM, see "T") the inhibitory effects of the NRTI-TPs were no longer observed, and the levels of full-length product were increased. Indeed, the dNTP concentration increase reduced the efficacy of these two NRTI-TPs to a similar extent as the addition of each drug individually (data not shown).

Next, we investigated whether Vpxmediated SAMHD1 degradation also abolishes the efficacy of multiple NRTIs when simultaneously applied to macrophages. For this test, primary human macrophages were again exposed to VLPs +/-Vpx and then simultaneously treated with AZT and ABC or ddC and TDF followed by transduction with HIV-1 D3. As shown in Figure 3B, combination NRTI treatment in macrophages exposed to VLPs +Vpx only reduced transduction efficiency to a similar degree as single NRTI treatment. Therefore, these data support that Vpx-mediated SAMHD1 degradation, which elevates all four dNTPs, efficiently counteracts the anti-viral efficacy of combination NRTI treatment commonly used in HAART

Effect of Vpx on HIV-1 sensitivity to NRTIs in activated CD4+ T cells - SAMHD1 expression levels directly influence cellular dNTP concentrations in non-dividing cells (12- 15). Next, we tested whether degradation of SAMHD1 in primary human activated CD4+ T cells, which are a dividing cell type, affects cellular dNTP concentrations and HIV-1 sensitivity to NRTIs. After treatment with VLPs +/-Vpx for 24 h, SAMHD1 expression levels decreased approximately 2-fold in the VLP +Vpx treated cells (Figure 4A). Cellular metabolites from these donors were also extracted and cellular dNTP concentrations were measured using a single nucleotide RT primer extension assay (10). Figure 4B shows that activated CD4+ T cells treated

with VLPs +Vpx contain 2.5-7.8 times higher dNTP levels than those treated with VLPs –Vpx. This elevation of cellular dNTPs in activated T cells is much smaller than the Vpx-mediated dNTP level elevation in macrophages (up to 33-fold, (12)). Exact nucleotide concentrations averaged from the three donors are shown in Table 1. Additionally, the transduction efficiency in T cells was not significantly altered by Vpx treatment (data not shown) whereas Vpx enhanced transduction in macrophages by 20- 30%. This is likely caused by the kinetic parameters of RT. The dNTPs found in macrophages are below the Km and Kd of RT (11), therefore the increase in dNTPs upon Vpx treatment greatly increases the rate of DNA synthesis (18). However, the 2.5-7.8-fold increase in dNTPs in T cells does not alter the rate of RT since the baseline dNTP levels are sufficient to saturate RT. To determine whether NRTI efficacy is affected by SAMHD1 degradation, activated T cells were again treated with VLPs, increasing concentrations of AZT, ABC, ddC, or TDF, and transduced with HIV-1 D3. As shown in Figure 4C-F, Vpx treatment decreased the efficacy of all four NRTIs tested, but to a much less degree as VLP +Vpx treated macrophages. These data support that SAMHD1 degradation only minimally affects HIV-1 sensitivity to NRTIs in activated CD4+ T cells.

SAMHD1 enzymatic activity toward AZT-TP and ddNTPs - Finally, we tested the possibility that SAMHD1 directly influences viral sensitivity to NRTIs by enzymatically hydrolyzing NRTI-TPs. Although our data demonstrate that expression of SAMHD1 maintains low dNTPs and allows for NRTIs to efficiently inhibit RT, it is possible that SAMHD1 hydrolyses NRTI-TPs at a slower rate than dNTPs. Therefore, determining whether NRTI-TPs are substrates for SAMHD1 may influence future drug design such that NRTI-TPs are at the highest possible concentration in the cell. To detect whether SAMHD1 has the ability to hydrolyze all four ddNTPs and AZT-TP, we incubated these nucleotides with purified recombinant human SAMHD1, shown in Figure 5A. When assayed using thin layer chromatography (TLC), recombinant GSTSAMHD1 had similar specific activity as

immunoprecipitated HA-SAMHD1 from 293FT cells, 50 versus 58 nM dA/ng SAMHD1/min, respectively (data not shown). Therefore, recombinant SAMHD1 was incubated separately with each substrate with or without the allosteric activator dGTP to ensure hydrolysis is not due to contaminating phosphatases in the protein preparation. As controls, SAMHD1 was also incubated with each canonical nucleotide. The remaining un-hydrolyzed nucleotide substrates in the triphosphate form were then separated and quantified with anion exchange HPLC (17,31). As shown in Figure 5B-E, hydrolysis of AZTTP or any of the ddNTPs could not be detected by this assay, but SAMHD1-mediated hydrolysis of all dNTPs could be detected indicating our protein is active. We cannot rule out that SAMHD1 may hydrolyze NRTI-TPs at a very slow rate since our assay is not sensitive enough to detect very low concentrations of nucleotides. Overall, these data suggest that for efficient hydrolysis to occur the 3' OH moiety on the ribose may be necessary for either SAMHD1 active site binding or for proper alignment of the α-phosphate with the catalytic residues. It also suggests that SAMHD1 does not significantly alter the concentration of the active form of NRTIs in the cell by direct hydrolysis of NRTITPs, and that the effect of SAMHD1 on NRTI efficacy results primarily from modulation of cellular dNTP concentrations.

Allosteric activation of SAMHD1 with ddGTP - Previous studies have shown that SAMHD1 is allosterically activated by dGTP (16,17); however, it has yet to be determined whether ddGTP is capable of activating SAMHD1. To test this, SAMHD1 was incubated with dATP as substrate in the presence of dGTP or ddGTP as activators, and the percentage of dATP remaining was quantified using HPLC. As shown in Figure 5F, ddGTP activated SAMHD1 to the same extent as dGTP. This result provides further insight into the specificity of the allosteric site of SAMHD1 and demonstrates that an interaction with a 3' OH moiety in the allosteric site of SAMHD1 is not necessary for activation, since ddGTP is fully capable of activating SAMHD1. More importantly, these data suggest that ddGTP, and possibly other dideoxyguanosine analogues, can modulate cellular dNTP concentrations by activating SAMHD1.

Discussion

Unwanted interactions of NRTIs with host molecules such as DNA polymerase γ, the mitochondrial DNA polymerase, can lead to off target effects (35-37). Since SAMHD1, the newly identified anti-viral host factor, interacts with and hydrolyzes cellular dNTPs, which chemically mimic NRTI-TPs, we tested whether SAMHD1 influences the anti-viral efficacy of NRTIs by directly hydrolyzing NRTI-TPs. With our HPLC-based assay we could not detect SAMHD1-mediated hydrolysis of ddNTPs or AZT-TP (Figure 5), however it is possible that there was minimal hydrolysis of NRTI-TPs that would require a more sensitive assay to be detected. These data suggest that SAMHD1 does not significantly alter the concentration of NRTI-TPs in the cells. Additionally, we show ddGTP can activate SAMHD1, which may result in further reduction of dNTPs when dideoxyguanosine analogues are applied to cells.

One challenge with competitive inhibitors is that the drug needs to be at a high enough concentration in the cell to out-compete the canonical cellular substrates. HIV-1 and HIV-2 primarily infect CD4+ T cells and macrophages, which have a large disparity in dNTP concentrations (10,11). Activated T cells are rapidly expanding and replicating their genomic DNA, whereas macrophages are nondividing, terminally differentiated cells, which do not undergo chromosomal DNA replication. As a consequence, dNTP concentrations in activated PBMCs range from 1-16 μM while dNTP concentrations in macrophages range from 20-70 nM (10,11). However, the dNTP concentrations in macrophages increase from 5- 33-fold upon Vpx-mediated degradation of SAMHD1 (12), which is likely similar to what occurs during HIV-2 infection. Aquaro et al. measured the EC50 of NRTIs for inhibition of HIV-1 infection in macrophages and PBMCs, and as expected PBMCs require a higher concentration of NRTIs to inhibit viral replication (reviewed in, (34,38)). This led us to test whether degradation of SAMHD1 impacts the efficacy of NRTIs in both macrophages and activated CD4+ T cells. Indeed, our data show that degradation of SAMHD1 by treatment with VLPs +Vpx significantly

reduces HIV-1 sensitivity to NRTIs in macrophages (Figure 2). This result was anticipated based on the previous study (38) and based on our biochemical data showing that increased dNTPs reduce AZT-TP and ddCTP inhibition of RT by active site competition. The reduction in drug efficacy observed in the cell culture experiments may also be influenced by competition for the active site of cellular kinases since NRTIs are not as efficiently phosphorylated as dNs (33). Additionally, when VLPs +Vpx were added to activated T cells, NRTI efficacy was decreased to a much lesser extent than VLP +Vpx treated macrophages (Figure 4). This likely results from the fact that the dNTP level is only minimally elevated by Vpx in activated T cells, compared to the Vpx-treated macrophages.

NRTIs are a key component in HAART that has been designed to treat patients with HIV-1. However, individuals infected with HIV-2 or dually infected with HIV-1 and HIV-2 also rely on HAART to maintain low viral loads. Therefore, it is essential to understand how differences in these viruses impact the efficacy of these inhibitors. This study focuses on the impact of SAMHD1 on the efficacy of NRTIs. SAMHD1 remains expressed in cells infected by HIV-1; however, HIV-2 expresses the accessory protein Vpx to target SAMHD1 for proteasomal degradation (13,19-24). Degradation of SAMHD1 upon VLP +Vpx treatment increases dNTP concentrations in macrophages (12) and activated CD4+ T cells (Figure 4). Overall, this study suggests that degradation of SAMHD1 directly decreases the anti-viral efficacy of NRTIs in macrophages by elevating cellular dNTPs suggesting that these drugs are not as effective at inhibiting HIV-2 infection in macrophages. Finally, this study further supports that the lack of Vpx in HIV-1 and the active site specificity of SAMHD1 to hydrolyze dNTPs more efficiently than NRTI-TPs synergistically contribute to the high efficacy of the currently available NRTIs against HIV-1.

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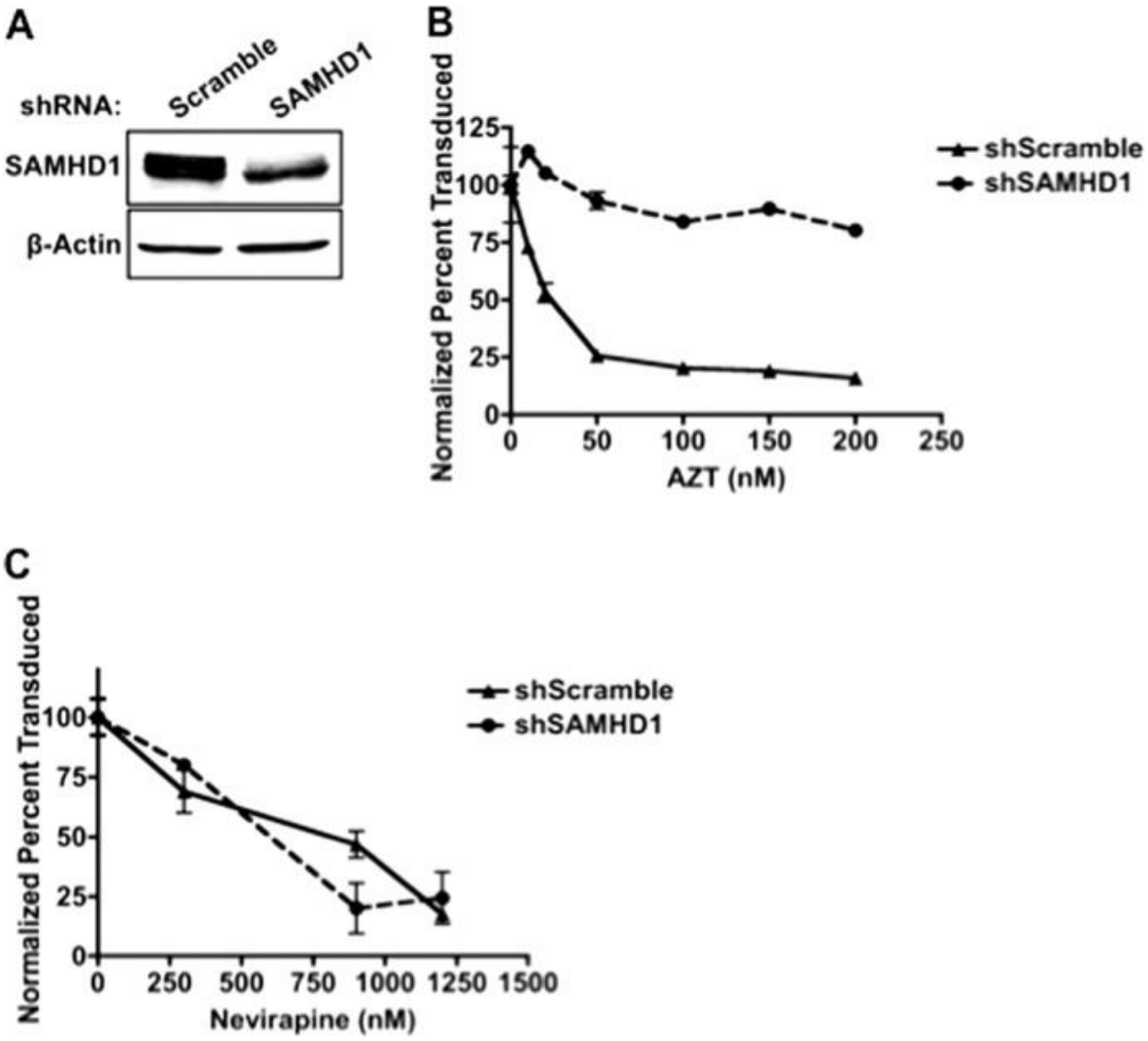

Figure 1. Knockdown of SAMHD1 decreases NRTI efficacy in THP1 cells. *A*, Western blot of SAMHD1 expression in differentiated THP1 cells with a scramble shRNA or a SAMHD1-specific shRNA. *B* and *C*, THP1 cell lines were differentiated, treated with increasing concentrations of AZT (*B*) or the NNRTI nevirapine (*C*), and transduced with HIV-1 D3. To determine transduction efficiency, cells were collected 48 hpt to measure GFP expression by flow cytometry (*error bars* represent the S.E., $n = 3$).

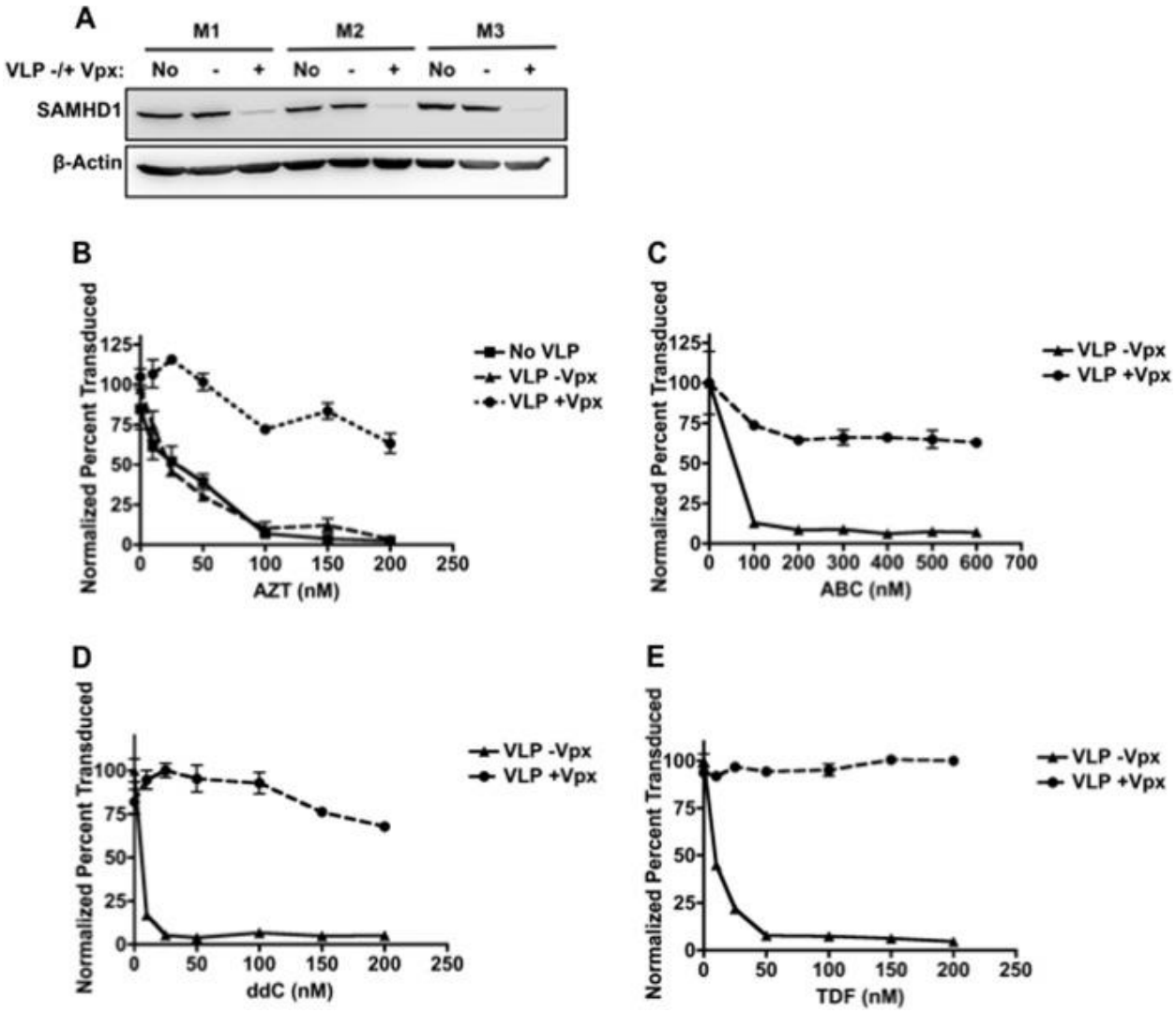

Figure 2. Vpx-mediated degradation of SAMHD1 decreases NRTI efficacy in macrophages. *A*, SAMHD1 expression level in three donors of macrophages (*M1*, *M2*, and *M3*) treated without VLPs or with VLPs ±Vpx for 24 h. *B–E*, macrophages were treated with VLPs for 24 h, treated with increasing concentrations of AZT (*B*), ABC (*C*), ddC (*D*), or TDF (*E*), and then transduced with HIV-1 D3. To determine transduction efficiency, cells were collected 7 dpt to measure GFP expression by flow cytometry (*error bars* represent the S.E., $n = 3$).

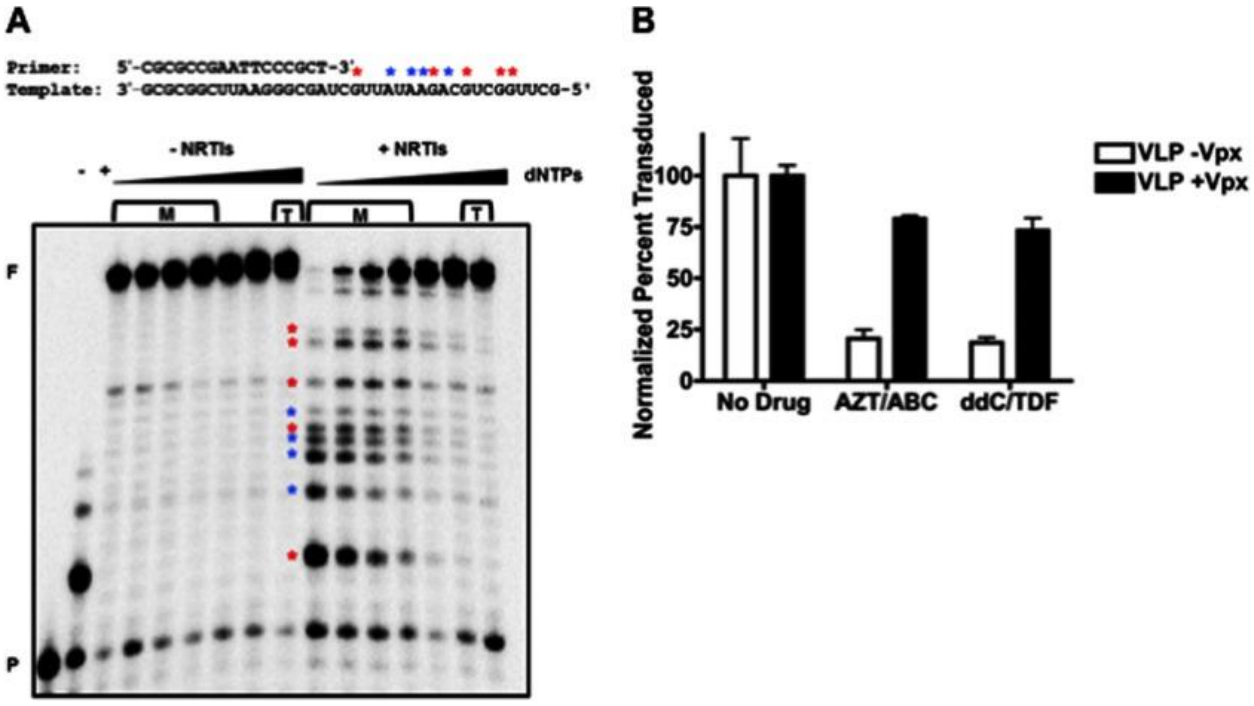

Figure 3:.Vpx-mediated degradation of SAMHD1 decreases the efficacy of combination NRTI treatment. *A*, purified HIV-1 RT protein was used to extend the indicated 5′ 32P-labeled primer/template with increasing dNTP concentrations (10 nm, 25 nm, 50 nm, 100 nm, 250 nm, 500 nm, and 1 μm) with or without a fixed concentration of AZT-TP and ddCTP. The dNTP concentrations found in macrophages and activated T cells are marked as *M* and *T*, respectively. The negative control (−) was without drug or dNTPs, and the positive control (+) was with drug and 1 nm dNTPs. *Asterisks* in *blue* and *red* indicate positions of chain termination for AZT-TP and ddCTP, respectively. *F*: fully extended 38-mer product; *P*: unextended 17-mer primer. *B*, VLP-treated macrophages were treated with 150 nm AZT/400 nm ABC or 150 nm ddC/200 nm TDF and transduced with HIV-1 D3. To determine transduction efficiency, cells were collected to measure GFP expression by flow cytometry (*error bars* represent the S.E., $n = 3$).

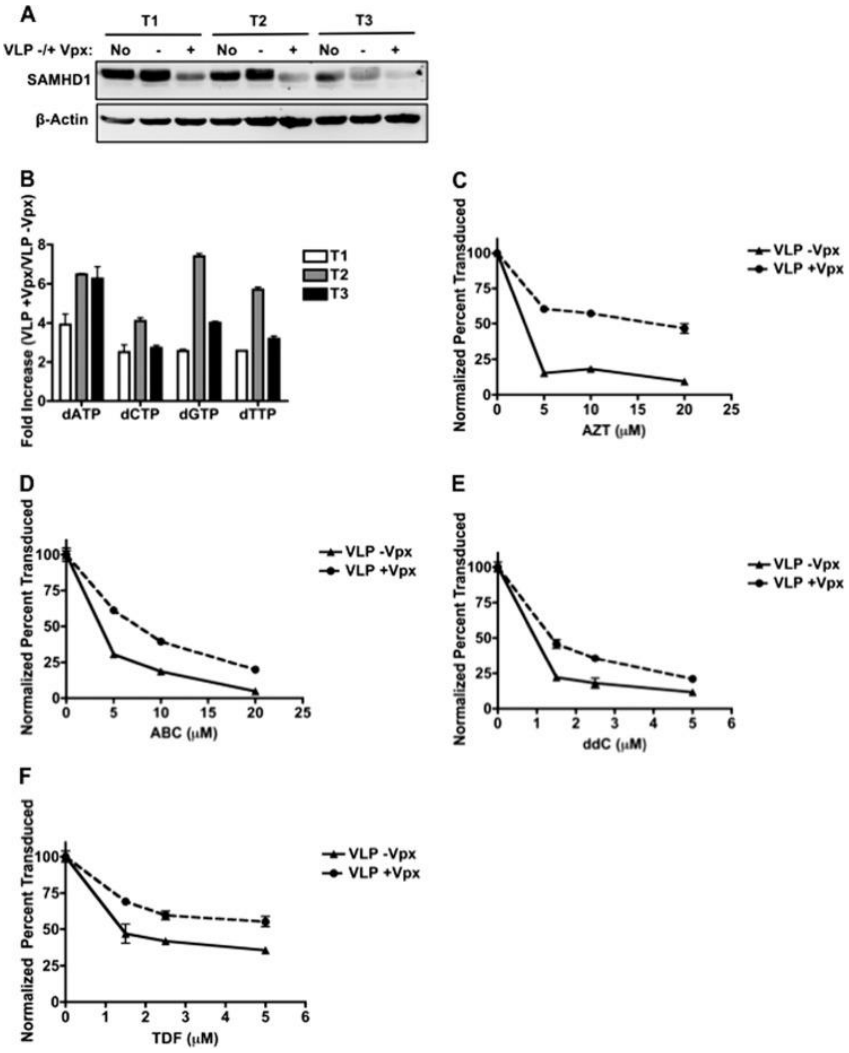

Figure 4. Vpx-mediated degradation of SAMHD1 decreases NRTI efficacy in activated T cells. *A*, SAMHD1 expression level in three donors of activated CD4+ T cells (*T1*, *T2*, and *T3*) treated without VLPs or with VLPs ±Vpx for 24 h. *B*, using the same three donors, dNTPs were collected and measured using a single nucleotide RT primer extension assay (10). -Fold increase of dNTPs in VLP +Vpx-treated cells when compared with VLP −Vpx-treated cells is shown (*error bars* represent the S.E., $n = 2$). *C–F*, activated CD4+ T cells were treated with VLPs ±Vpx for 24 h, treated with increasing concentrations of AZT (*C*), ABC (*D*), ddC (*E*), or TDF (*F*), and then transduced with HIV-1 D3. To determine transduction efficiency, cells were collected to measure GFP expression by flow cytometry (*error bars* represent the S.E., $n = 3$).

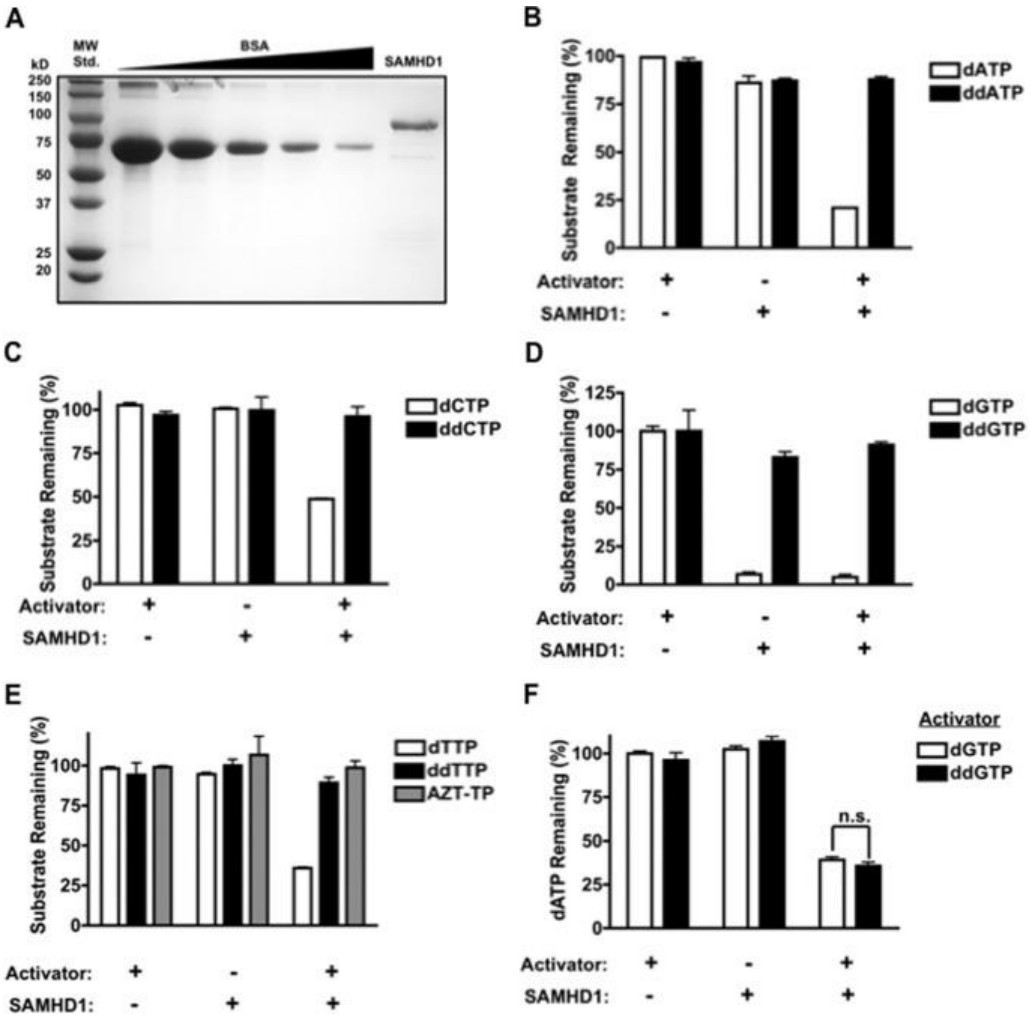

Figure 5. SAMHD1 enzymatic activity toward ddNTPs and allosteric activation with ddGTP. *A*, recombinant SAMHD1 and a standard curve of bovine serum albumin (8 μg-0.5 μg, BSA) were run on SDS-PAGE and stained with Coomassie Blue. *MW Std.*, molecular weight standard.*B–E*, purified SAMHD1 was incubated with the indicated nucleotide in the presence or absence of 100 μm dGTP as an allosteric activator. Nucleotide substrates remaining in the triphosphate form were separated and quantified using HPLC (*error bars* represent the S.E., n = 3). *F*, SAMHD1 was incubated with 1 mm dATP and 500 μm of dGTP or ddGTP. The quantity of dATP remaining after incubation was measured using HPLC and quantified as described under "Experimental Procedures" (p, 0.2599). Statistical significance was measured using an unpaired t test with a Welch's correction, n = 3.

Supplementary Figure 1.

A

dNTP (μM)	No VLP	VLP -Vpx	VLP +Vpx
dATP	1.83 ± 0.21	2.41 ± 0.6	13.33 ± 4.64
dCTP	1.47 ± 0.45	1.54 ± 0.54	5.07 ± 2.83
dGTP	1.71 ± 0.19	2.18 ± 0.46	10.57 ± 5.92
dTTP	2.15 ± 0.26	2.57 ± 0.50	9.88 ± 3.87

Table 1. Degradation of SAMHD1 in activated CD4$^+$ T cells increases cellular dNTPs. dNTP concentrations in three donors of activated CD4$^+$ T cells were measured using a single nucleotide RT primer extension assay. Error was calculated using the standard error of the mean for the average dNTP concentration in each donor.

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Chapter V: SAMHD1 controls cell cycle status, apoptosis and HIV-1 infection in monocytic THP-1 cells.

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Contributions:

M.Daly characterized the SAMHD1 KO THP-1 and overexpression clones prior to all experimental use including determination of SAMHD1 expression, dNTP levels, adequate differentiation conditions and HIV infectivity (data not shown in manuscript). M.Daly contributed Figure 1 (Part A+D), Figure 4 (parts D+E), Supplementary Figure 1 (part A+B) and co-wrote the manuscript.

Abstract

SAMHD1 limits HIV-1 infection in non-dividing myeloid cells by decreasing intracellular dNTP pools. HIV-1 restriction by SAMHD1 in these cells likely prevents activation of antiviral immune responses and modulates viral pathogenesis, thus highlighting a critical role of SAMHD1 in HIV-1 physiopathology. Here, we explored the function of SAMHD1 in regulating cell proliferation, cell cycle progression and apoptosis in monocytic THP-1 cells. Using the CRISPR/Cas9 technology, we generated THP-1 cells with stable SAMHD1 knockout. We found that silencing of SAMHD1 in cycling cells stimulates cell proliferation, redistributes cell cycle population in the G_1/G_0 phase and reduces apoptosis. These alterations correlated with increased dNTP levels and more efficient HIV-1 infection in dividing SAMHD1 knockout cells relative to control. Our results suggest that SAMHD1, through its dNTPase activity, affects cell proliferation, cell cycle distribution and apoptosis, and emphasize a key role of SAMHD1 in the interplay between cell cycle regulation and HIV-1 infection.

Keywords

SAMHD1; Monocytic cells; Gene knockout; HIV-1; dNTP; Restriction; Cell cycle; Apoptosis

1. Introduction

SAM domain- and HD domain-containing protein 1 (SAMHD1) is the first deoxynucleoside triphosphate triphosphohydrolase (dNTPase) identified in mammalian cells (Goldstone et al., 2011). SAMHD1 induces the hydrolysis of dNTPs and, in concert with cellular ribonucleotide reductase, functions as a key regulator of intracellular dNTP homeostasis. In 2011, two laboratories independently identified SAMHD1 as a host restriction factor inhibiting human immunodeficiency virus type 1 (HIV-1) infection in non-dividing myeloid cells (Hrecka et al., 2011 and Laguette et al., 2011). The viral protein X (Vpx) uniquely expressed by lentiviruses such as HIV-2 and many strains of simian immunodeficiency virus, but not by HIV-1, has been

shown to restore HIV-1 infection in myeloid cells (Guyader et al., 1989 and Yu et al., 1991). The underlying molecular mechanisms remained unknown until the discovery that Vpx targets SAMHD1 for proteasomal degradation, thus counteracting its restriction activity and supporting HIV-1 infection (Hrecka et al., 2011 and Laguette et al., 2011). We and others showed that SAMHD1 dNTPase activity is responsible for the extremely low dNTP concentrations associated with the kinetic delay in HIV-1 reverse transcription, and that Vpx accelerates the viral DNA synthesis by elevating cellular dNTP levels in myeloid cells and resting $CD4^+$ T lymphocytes, thus revealing the mechanistic and regulatory links among SAMHD1, Vpx, cellular dNTPs, and viral reverse transcription kinetics (Baldauf et al., 2012, Kim et al., 2012, Lahouassa et al., 2012 and St Gelais et al., 2012). Besides its dNTP hydrolase function, SAMHD1 harbors RNase and nuclease activities, which have been postulated to possibly contribute to HIV-1 restriction (Beloglazova et al., 2013, Choi et al., 2015 and Ryoo et al., 2014), although these results require further validation.

In addition to the role of SAMHD1 as a host restriction factor, mutations in the *SAMHD1*gene have been linked to a genetic immune disorder called Aicardi-Goutières Syndrome (AGS) (Rice et al., 2009), as well as several types of cancer, of both solid and hematological origins [reviewed in (Kohnken et al., 2015)]. These accumulating lines of evidence suggest the involvement of SAMHD1 in the innate immune response and cancer development through the control of dNTP homeostasis. In the last few years, intense efforts have been carried out in order to define the mechanisms by which SAMHD1 interferes with HIV-1 infection in non-dividing cells (Wu, 2013), and unravel its role in immunological diseases and cancer development. However, the physiological functions of SAMHD1 remain to be fully defined, and elucidation of the underlying mechanisms would help the development of potential therapeutic approaches in the context of HIV-1, AGS and cancer.

Here, we developed a monocytic THP-1 cell line model presenting stable knockout (KO) of the *SAMHD1* gene by using the CRISPR/Cas9 genome editing technology. We aimed to characterize the phenotype of THP-1 cells lacking SAMHD1 protein in comparison to control cells expressing the endogenous and functional protein. We focused on the effects of SAMHD1 on cell proliferation, cell cycle regulation and cell death, and their potential correlation with HIV-1 restriction. We found that *SAMHD1* silencing leads to increased cell growth, perturbation of the cell cycle and reduced susceptibility to apoptosis. Moreover, we observed increased dNTP levels and enhanced HIV-1 infection in dividing SAMHD1 KO THP-1 cells relative to control cells, thus confirming the role of SAMHD1 in the control of HIV-1 life cycle in myeloid cells. Our results shed light on a functional interplay between SAMHD1-mediated regulation of cell cycle, apoptosis and HIV-1 infection through its dNTPase activity.

2. Results

2.1. Stable silencing of the SAMHD1 gene in THP-1 cells

THP-1 cells have been widely used as a cell line model to investigate the functions of primary myeloid-lineage cells such as monocytes, macrophages and dendritic cells (Auwerx, 1991, Berges et al., 2005 and Chanput et al., 2014). Here, we employed the CRISPR/Cas9 technology to knockout *SAMHD1* in THP-1 cells. We chose the THP-1 cell line for our model system because, opposed to other monocytic cell lines such as U937 cells lacking endogenous SAMHD1 expression, THP-1 cells express similar levels of SAMHD1 compared to primary myeloid cells (Laguette et al., 2011). Two single guide RNAs (gRNA 1 and gRNA 2 indicated in Fig. 1(A)) targeting unique sequences in exon 1 of the *SAMHD1* gene were designed and cloned into a lentiviral vector (Sanjana et al., 2014 and Shalem et al., 2014). Polyclonal undifferentiated THP-1 cells were transduced with a lentiviral vector expressing gRNA 1 or gRNA 2, Cas9 and a puromycin resistance marker (Shalem et al., 2014). To assess the ability of the gRNAs to cleave *SAMHD1*, we used the Surveyor nuclease assay (Guschin et al., 2010 and Ran et al., 2013).

Compared to untargeted cells, cleavage products were detected only in genomic DNA from polyclonal THP-1 cells transduced with lentiviral vector containing either gRNA 1 or 2 (Fig. 1(B), shown by arrows), indicating that the gRNAs successfully targeted the region of interest and, therefore, are valid candidates for knocking out *SAMHD1*. Immunoblotting analysis confirmed the loss of SAMHD1 expression in three cell clones transduced with two different gRNAs targeting *SAMHD1* (KO), while endogenous SAMHD1 expression was detected in the THP-1 clones transduced with the control vector (Fig. 1(C) and Supplementary Fig. 1A).

As SAMHD1 is a key regulator of intracellular dNTP homeostasis (Ballana and Este, 2015), we analyzed dNTP levels in non-differentiated SAMHD1 KO and control cells by using our previously described dNTP assay (Diamond et al., 2004). Intracellular dNTP pools increased 3- to 6-fold in SAMHD1 KO compared to control cells, as shown in Fig. 1(D) (KO clone 1, derived from gRNA 2, and control clone 1, referred respectively as KO and control from here on) and Supplementary Fig. 1B. Our data are consistent with previously published results showing a significant increase in the dNTP levels in primary human macrophages or dendritic cells in which efficient SAMHD1 degradation was induced by Vpx (Hollenbaugh et al., 2014; Kim et al., 2012 and St Gelais et al., 2012), thus further confirming that the complete silencing of SAMHD1 expression in KO cells leads to up-regulation of the intracellular dNTP pool.

2.2. Silencing of SAMHD1 affects cell proliferation and alters cell cycle status

Overexpression of SAMHD1 has been described to reduce the proliferation of HeLa cells and the lung cancer cell line A549 (Clifford et al., 2014 and Wang et al., 2014). To investigate the effect of SAMHD1 on THP-1 cell growth, we analyzed the proliferation of non-differentiated control and KO cells by trypan blue exclusion and 3-(4,5-dimethylthiazol-2-yl)−5-(3-carboxymethoxyphenyl)−2-(4-sulfophenyl)−2 h-tetrazolium (MTS) assays. By performing a time course experiment, we observed enhanced growth rate and viability of KO cells compared to control cells, with a significant increase of the number of live cells at days 5 and 7 after seeding

(1.6 and 1.4-fold, respectively, Fig. 2(A)), while a significant increase of cell proliferation and metabolism (1.3-fold) was observed from day 3 to day 7 (Fig. 2(B)). These results prompted us to investigate whether SAMHD1 may induce perturbation of the cell cycle. Flow cytometry analysis performed in asynchronous cells over a period of 7 days showed that knockout of SAMHD1 was associated with significantly increased G_1/G_0 cell population in comparison to control cells, while a decrease was observed in the fraction of cells in G_2/M phase (Fig. 2(C)). A similar cell cycle profile was obtained in cells synchronized in G_1phase by serum starvation (data not shown), further suggesting that SAMHD1 delays cell cycle progression most likely through accumulation of the cells in G_2/M. Notably, additional two SAMHD1 KO cell clones (derived from gRNA 1 or 2) showed similar phenotypes compared to control cells (Supplementary Fig. 1C–D), thus confirming that the effects on cell proliferation and cell cycle status can be specifically ascribed to SAMHD1 silencing. Although the investigation of the molecular mechanisms associated with these effects is beyond the scope of this study, these data suggest that SAMHD1 negatively impacts cell cycle progression, leading to reduced cell growth and proliferation.

2.3. Silencing of SAMHD1 results in reduced spontaneous activation of apoptosis

We next evaluated whether reduced proliferation of control cells is correlated with increased cell death. We analyzed apoptosis induction by Annexin-V staining, which allowed us to distinguish cells in early and late apoptosis due to the exposure of phosphatidylserine residues on the cell membrane, an event occurring at early stages during apoptotic cell death (Verhoven et al., 1995). As shown in Fig. 3(A) andSupplementary Fig. 2A, a higher percentage of early or late apoptotic cells was observed, respectively, at 1 or 3 days post-seeding in SAMHD1 expressing control cells compared to KO cells, consistently with more pronounced cell proliferation (Fig. 2(B) andSupplementary Fig. 1C) and G_1/G_0 accumulation (Fig. 2(C) and Supplementary Fig. 1D) detected in KO clones. In line with the data in Fig. 2(A) showing more robust cell viability, flow cytometry analysis of apoptosis demonstrated a higher percentage of the live KO cell population

compared to control cells (Fig. 3(A), left panel). Moreover, cells expressing SAMHD1 showed increased activation of caspase 3, as demonstrated by detection of cleaved active forms of caspase 3 and cleavage of poly (ADP-ribose) polymerase (PARP), a known target of active caspase 3 and marker of apoptosis (Boulares et al., 1999) (Fig. 3(B) and Supplementary Fig. 2B), and by direct measurement of caspase 3/7 activities using a luminescence-based assay (Fig. 3(C) andSupplementary Fig. 2C). Compared to control cells, SAMHD1 KO cells showed very low or absent caspase activation (e.g., 7-fold decrease at day 3 in Fig. 3(C)). These data suggest that SAMHD1 silencing may render the cells less prone to apoptosis compared to normal cells.

2.4. Effect of SAMHD1 knockout and overexpression on HIV-1 infection in THP-1 cells

SAMHD1 is a known restriction factor of HIV-1 infection in non-dividing cells such as primary monocytes, dendritic cells, macrophages and resting $CD4^+$ T-cells, where SAMHD1 is highly expressed (Baldauf et al., 2012, Berger et al., 2012, Hrecka et al., 2011 and Laguette et al., 2011). SAMHD1-mediated HIV-1 restriction in phorbol 12-myristate 13-acetate (PMA)-differentiated, non-dividing macrophage-like cells (such as THP-1 or U937 cell lines) has been previously reported (Laguette et al., 2011 and Lahouassa et al., 2012), but its effect on virus infection efficiency in dividing monocytic cell lines has not been carefully characterized. To explore this aspect and identify a potential link between SAMHD1 restriction activity and its effects on cell proliferation, cell cycle and apoptosis, we first analyzed SAMHD1 expression in control and KO cells differentiated or not with PMA treatment. Immunoblotting analysis showed a modest increase of SAMHD1 protein level in PMA-differentiated non-cycling control cells, in line with our published results (St Gelais et al., 2014) (Fig. 4(A)). Next, non-differentiated and differentiated THP-1 control and SAMHD1 KO cells were infected with a single-cycle luciferase reporter HIV-1 pseudotyped with the vesicular stomatitis virus protein G (HIV-1-Luc/VSV-G) (Wang et al., 2016). Luciferase activity was measured at 24 and 48 h post-infection (hpi) as an indication of infection efficiency. At 24 hpi, cycling KO cells were more susceptible to HIV-1

infection compared to control cells (5-fold increase,Fig. 4(B) left panel), indicating that SAMHD1 is able to counteract HIV-1 infection in dividing THP-1 monocytic cells. At 48 hpi, HIV-1 infection efficiency was similar in the two cell lines, irrespective of SAMHD1 expression. Further investigation is required to unravel the mechanism responsible for the loss of SAMHD1 restriction activity in these cells at this time point. Conversely, in non-cycling SAMHD1 KO cells, HIV-1 infection was more efficient at both time points (14- and 20-fold increase at 24 and 48 hpi, respectively) (Fig. 4(B), right panel), confirming that silencing of SAMHD1 in THP-1 cells results in the expected HIV-1 restriction phenotype as described in published studies (Laguette et al., 2011, Lahouassa et al., 2012 and St Gelais et al., 2014).

To confirm that the effects on dNTP intracellular concentration (Fig. 1(D)) and HIV-1 infection (Fig. 4(B)) observed in KO cells were due to the absence of SAMHD1 expression, the mutated *SAMHD1* gene resistant to the CRISPR/Cas9 targeting was re-introduced into the KO cells by transducing the KO cell line derived from gRNA 2 with either control vector (Lvx) or SAMHD1-expressing (SAM) lentiviruses. The clones (six for Lvx and four for the SAMHD1 expression vector) were analyzed for SAMHD1 expression after differentiation with PMA (Fig. 4(C)). As expected, SAMHD1 protein was not detected in the six Lvx clones, whereas four SAM clones displayed robust SAMHD1 expression. Notably, SAMHD1 expression levels in the SAM clones were significantly higher than those in THP-1 parental and control cells (Fig. 4(C) and data not shown). Overexpression of SAMHD1 reversed the increased dNTP concentration phenotype observed in the KO and Lvx cells (Fig. 4(D)). Interestingly, dATP, dGTP and dTTP levels in the SAMHD1 cells were lower than those detected in control cells (Fig. 4(D)). This could be due to the increased expression of SAMHD1 in the SAM clones compared to the control cells.

Furthermore, we investigated the effect of SAMHD1 overexpression on HIV-1 infection of differentiated cells. PMA-treated THP-1 cells were infected with VSV-G-pseudotyped HIV-1-GFP (Diamond et al., 2004) and analyzed for GFP expression by flow cytometry at 72 hpi. As

shown in Fig. 4(E), KO and Lvx THP-1 cells lacking SAMHD1 displayed elevated HIV-1 infection compared to control cells (7- and 5-fold increase, respectively), correlating the loss of SAMHD1 with enhanced HIV-1 replication. HIV-1 infection was limited in SAM cells, which were also more effective at HIV-1 restriction than the control cells (Fig. 4(E)), consistently with the higher SAMHD1 expression and lower dNTP concentration observed in the SAM cells (Fig. 4(C) and (D)). These data indicate the tight interplay among SAMHD1 expression levels, dNTP concentrations, and HIV-1 infection efficiency, which we and others have previously observed in primary human macrophages or dendritic cells (Baldauf et al., 2012, Kim et al., 2012, Laguette et al., 2011, Lahouassa et al., 2012 and St Gelais et al., 2012). Lastly, we sought to test whether the SAM clones were sensitive to Vpx-mediated SAMHD1 degradation and the treatment with deoxynucleosides (dNs), which independently elevate intracellular dNTP levels through the salvage pathway of dNTP synthesis (Lahouassa et al., 2012). We used a combined Vpx/dNs treatment as neither treatment alone resulted in a significant increase of HIV-1 infection efficiency (data not shown), most likely due to the very high expression levels of SAMHD1 in the SAM clones. As expected, SAMHD1-mediated suppression of HIV-1 infection of SAM cells was rescued by the combined Vpx/dNs treatment (Fig. 4(E)).

Taken together, these results show that SAMHD1 knockout and overexpression in THP-1 cells modulate HIV-1 infection by regulating the intracellular dNTP concentration, thus biochemically and virologically mimicking what is observed with primary monocyte-derived macrophages or dendritic cells, and can be a convenient tool to further study the precise mechanisms of SAMHD1-mediated retroviral restriction.

3. Discussion

SAMHD1 is a cellular restriction factor that limits HIV-1 infection in non-dividing myeloid cells and resting $CD4^+$ T cells, by inducing the hydrolysis of dNTPs, which inhibits the viral reverse transcription process (Baldauf et al., 2012 and Lahouassa et al., 2012). Non-productive infection

of myeloid cells by SAMHD1 likely prevents activation of anti-viral immune responses, which may allow HIV-1 to escape immune recognition and establish persistent infection (Wu, 2012). Therefore, complete elucidation of the physiological functions of SAMHD1 in myeloid cells is important for the development of potential anti-viral approaches.

In this study, we generated a stable SAMHD1 KO THP-1 cell line to explore the effect of silencing SAMHD1 on cell proliferation, cell-cycle progression, and apoptosis. Beside its role in HIV-1 restriction, SAMHD1 is known to influence the proliferation of several cell types by regulating dNTP homeostasis (Kohnken et al., 2015). We characterized the phenotype of dividing SAMHD1 KO THP-1 cells in comparison to control cells, and found that SAMHD1 reduces cell proliferation and metabolism, in line with previous published work showing that overexpression of SAMHD1 negatively affects the proliferation of cancer cells (Clifford et al., 2014 and Wang et al., 2014). Moreover, we observed that silencing of SAMHD1 redistributes cells mainly in the G_1/G_0 phase of the cell cycle. Our results are consistent with published data showing that SAMHD1 knock-down in proliferating human fibroblast cell lines (Franzolin et al., 2013) and that SAMHD1 mutations in fibroblasts from AGS patients (Kretschmer et al., 2015) result in accumulation of the cells in G_1, decreased percentage of cells in G_2 and increase of the intracellular dNTP pools. In addition to its effect on cell cycle progression, we found that SAMHD1 regulates apoptosis. Indeed, KO cells appear to be more resistant to spontaneous apoptotic cell death relative to control cells, as demonstrated by the reduced activation of caspase 3/7 and absent cleavage of the apoptotic marker PARP. Importantly, the effects of SAMHD1 silencing on cell growth, proliferation, cell cycle progression and apoptosis induction were observed in an additional control and two KO clones, thus suggesting that the phenotype described is unlikely due to off-target effects of the CRISPR/Cas9 system, and further confirming a key role of SAMHD1 in these processes.

Notably, changes in cell cycle progression were consistently associated with altered expression of *cyclin D3*. Our unpublished results revealed that the *cyclin D3* mRNA level is significantly down-regulated in KO cells compared to control cells (data not shown). This data is consistent with accumulation of SAMHD1 KO cells in G_1/G_0, as cyclin D3 controls cell cycle progression at G_1/S phase (Bartkova et al., 1998 and Herzinger and Reed, 1998). Further investigation will help elucidate a potential interplay of cyclin D3 with SAMHD1-mediated control of cell proliferation.

We found that disturbances in cell cycle progression induced by SAMHD1 silencing are associated with increased intracellular dNTP concentrations and more productive HIV-1 infection of KO versus control cells. Previous studies from our and other groups showed that overexpressed SAMHD1 does not restrict HIV-1 infection in dividing HeLa and HEK293T cells (St Gelais et al., 2012, St Gelais et al., 2014 and Welbourn and Strebel, 2016). Here we provide first evidence that SAMHD1 limits HIV-1 infection in actively proliferating THP-1 cells, and that the absence of SAMHD1 renders the cells more susceptible to HIV-1 infection. Increased HIV-1 infection efficiency could also be detected in differentiated cells, thus confirming the established HIV-1 restriction ability of SAMHD1 in non-dividing cells (Lahouassa et al., 2012), and further validating our THP-1 SAMHD1 KO model as a useful tool to study SAMHD1 functions in monocytic cells. Of note, while the enhancement of HIV-1 infection in differentiated KO cells is detected at both time points tested (24 and 48 hpi), we observed similar viral infection efficiency in non-differentiated control and SAMHD1 KO cells at 48 hpi. We exclude the possibility that this effect could be due to increased SAMHD1 T592 phosphorylation status, which is known to negatively regulate its HIV-1 restriction activity (Cribier et al., 2013 and White et al., 2013), at 48 hpi (data not shown). Further analysis is needed to identify the responsible mechanisms. Importantly, by performing SAMHD1 knock-in experiments, we could reverse the phenotype observed in KO cells, confirming that the increase in dNTP levels and HIV-1 infection efficiency was mainly due to the absence of SAMHD1. Moreover, in cells overexpressing SAMHD1, its

expression or activity could be impaired by co-treatment with Vpx and dNs, which are known to counteract SAMHD1-mediated HIV-1 restriction (Laguette et al., 2011 and Lahouassa et al., 2012). Of note, SAMHD1 overexpressing cells showed even lower dNTP concentrations and more efficient HIV-1 restriction compared to control cells, which could be due to different expression levels of SAMHD1 protein in the two cell lines.

Correlation between cell cycle progression and SAMHD1 expression levels and its HIV restriction function remains to be confirmed (Franzolin et al., 2013, Kretschmer et al., 2015, Pauls et al., 2014 and Yan et al., 2015). A complete picture of the mechanisms by which SAMHD1 influences cell cycle regulation is still missing, and it would be interesting to further investigate how cell cycle regulation affects its HIV-1 restriction activity in dividing cells, as well as to identify the role of cell cycle-related proteins during retroviral infection in myeloid cells.

In summary, by using a novel THP-1-derived cell model, we suggest that SAMHD1 can act as a regulator of the cell cycle and apoptosis-inducing factor in cycling THP-1 monocytic cells. These effects correlated with HIV-1 restriction, thus proposing a functional link between cell cycle control and HIV-1 restriction by SAMHD1. The new THP-1 cell model that we generated can expedite preliminary *in vitro* studies and future *in vivo* translational investigations, thus helping elucidate the cellular functions of SAMHD1 and mechanisms of retroviral restriction.

4. Materials and methods

4.1. Generation of SAMHD1 knockout THP-1 cells

Two single guide RNAs (gRNAs) targeting unique sequences of the SAMHD1 gene were designed and cloned into the lentiCRISPR v1 plasmid, which contains a puromycin resistance cassette and Cas9 nuclease (Sanjana et al., 2014 and Shalem et al., 2014). Lentiviral vectors were produced by co-transfection of polyclonal THP-1 cells with the lentiCRISPR v1/gRNA, d8.74 and pMD2 plasmids using the cationic polymer polyethylenimine (PEI) method (Boussif et al.,

1995). Viral supernatant was collected and used to transduce polyclonal undifferentiated THP-1 cells. Both gRNA constructs were functionally tested for their ability to mediate genomic insertion, deletion or inversions by isolating the genomic DNA from stably transduced and selected polyclonal population of THP-1 cells and performing a Surveyor nuclease assay as per manufacturer's instructions (Guschin et al., 2010 and Ran et al., 2013). Puromycin resistant cells were selected from each gRNA vector transduction, and then sorted by fluorescence-activated cell sorting (FACS) into 96-well plates for clonal expansion.

4.2. Generation of SAMHD1 overexpressing cell line

Silent mutations in the *SAMHD1* gene were introduced by site directed mutagenesis (QuikChange Lightning kit, Agilent Genomics) to make it resistant to the gRNA 2-mediated CRISPR/Cas9 cleavage. The following primers were used: 5′-GAAGCTGATTGGTCACCTGGACTAGAACTCCATCCCGACTAC-3′ and 5′-GTAGTCGGGATGGAGTTCTAGTCCAGGTGACCAATCAGCTTC-3′. This Cas9 resistant *SAMHD1* gene was cloned into LVX-IRES-mCherry vector (Clontech). Lentiviral vectors containing either LVX-IRES-mCherry control (Lvx, expressing only mCherry) or LVX-SAMHD1-IRES-mCherry (SAM, expressing SAMHD1 and mCherry) were produced by co-transfection of HEK293FT cells using the PEI method (Boussif et al., 1995). Viral supernatant was used to transduce the gRNA 2 KO cells. The mCherry expressing THP-1 cells were single-cell sorted by FACS into 96-well plates and clonally expanded.

4.3. Cell culture

THP-1-derived control, SAMHD1 KO, Lvx and SAM cell clones were maintained in RPMI-1640 (ATCC), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 µg/ml puromycin at 37 °C, 5% CO_2. To induce differentiation into non-dividing, macrophage-like cells, cells were treated with 100 ng/ml PMA for 24 or 72 h.

HEK293FT cells were grown in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin. GHOST/X4/R5 cells used for virus titration purposes were cultured as described (Wang et al., 2016).

4.4. Live cell counting

Control or SAMHD1 KO THP-1 cells (2.5×10^4 cells per well) were seeded in four replicates in 96-well plate in 100 µl of culture media. At the indicated time points, trypan blue was added to each well and live cell numbers were counted.

4.5. MTS assay for cell proliferation

Cell proliferation was measured according to the colorimetric CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). Control or SAMHD1 KO THP-1 cells (2.5×10^4 cells per well) were seeded in four replicates in 96-well plate in 100 µl of culture media. Wells containing only media were used for background measurement. At the indicated time points, plates were incubated for 1 h at 37 °C with 20 µl of MTS reagent and absorbance at 490 and 690 nm (specific and non-specific readings, respectively) was read with a plate reader. Cell proliferation was calculated as the average of 490 nm absorbance values, corrected for non-specific and background readings, of four replicates per sample.

4.6. Cell cycle analysis by propidium iodide staining

Control and SAMHD1 KO THP-1 cells were seeded, in triplicate, in 12-well plates at a density of 2×10^5 cells per well. At day 0, 1, 3, 5 and 7 after seeding, cells were counted, harvested, fixed in 70% ice-cold ethanol, and stained with Guava Cell Cycle Reagent (EMD Millipore), according to the manufacturer's instructions. Cell cycle data was then acquired on the Guava flow cytometer instrument and analyzed using Cytosoft 4.2.

4.7. Detection of apoptosis by Annexin-V staining

Control and SAMHD1 KO THP-1 cells were seeded as described for the cell cycle analysis. At day 0, 1, 3, 5 and 7 after seeding, cells were counted, washed in ice-cold PBS and resuspended in 1X Binding Buffer at a concentration of 1×10^6 cells/ml. Cells were then stained with PE-Annexin V and 7-Amino-Actinomycin (7-AAD) using the PE Annexin V Apoptosis Detection Kit I (BD Pharmingen), according to the manufacturer's instructions. Apoptosis data was acquired on the Guava flow cytometer, data analysis and gating was performed using FlowJo software.

4.8. Caspase 3/7 activation assay

Control and SAMHD1 KO THP-1 cells (5×10^5 per well) were seeded in 6-well plates. At day 0, 1, 3, 5 and 7 after seeding, cells were counted and 1×10^4 cells per well were transferred into a 96-well plate. Caspase 3/7 activity was determined using the luminescent Caspase-Glo 3/7 assay kit (Promega), according to manufacturer's instructions. Briefly, plates were incubated for 1 h at room temperature with 100 µl/well of Caspase-Glo 3/7 reagent, and luciferase activity was measured with a plate reader (Perkin Elmer). Wells containing only cell culture media were used to measure background luminescence, which was subtracted from experimental values.

4.9. Immunoblotting analysis

Cells were harvested, washed with PBS and lysed in cell lysis buffer (Cell Signaling) containing protease inhibitor (Sigma-Aldrich) as described (Wang et al., 2016). Cell extracts were resolved by SDS-PAGE and subjected to immunoblotting analysis using the following antibodies: rabbit polyclonal anti-SAMHD1 antibody (ProSci, #1224), rabbit polyclonal anti-caspase 3 antibody (Cell Signaling, #9662 S), rabbit polyclonal anti-PARP antibody (Cell Signaling, #9542). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) detection (AbD serotec) was used as loading control. Immunoblotting images were captured and analyzed by the Luminescent Image analyzer (LAS 4000) as previously described (Wang et al., 2016).

4.10. HIV-1 production and infection assays

Single cycle, luciferase reporter HIV-1-Luc/VSV-G was produced and titrated as previously described (Wang et al., 2016). Control or SAMHD1 KO THP-1 cell lines were infected with HIV-1-Luc/VSV-G at a multiplicity of infection (MOI) of 1 infectious unit per cell and infection efficiency was determined by measuring luciferase activity at 24 and 48 hpi using a luciferase reporter kit (Promega), according to manufacturer's instructions. Luciferase values were normalized to total protein concentration determined by bicinchoninic acid assay (BCA, Pierce). VSV-G pseudotyped D3HIV-GFP virus was produced as previously described with slight modification (Diamond et al., 2004). Briefly, HEK293FT cells were co-transfected with VSV-G protein and D3HIV-GFP, in which *env*and *nef* genes are deleted and replaced with GFP. Viral supernatant was collected at 48 hpi and concentrated (~200-fold) by ultracentrifugation at 22,000 rpm for 2 h at 4 °C. All PMA-differentiated THP-1 cells were infected with equal amounts of D3 vector by spinfection at 400 g for 30 min Cells were analyzed for GFP expression 72 hpi by flow cytometry (Miltenyi MACSQuant).

4.11. Intracellular dNTP measurement

Cellular dNTP levels were determined by a single nucleotide RT incorporation assay as previously described (Diamond et al., 2004). Four distinct 19-mer DNA templates, each with a distinct nucleotide (N) at the 5′ end (5′-NTGGCGCCCGAACAGGGAC-3′), were separately annealed to an 18-mer primer (5′-GTCCCTGTTCGGGCGCCA-3), 32 P-labelled at its 5′ end. Reactions contained 200 fmol template/primer, 4 µl of purified RT (HIV-1 HXB2), 25 mM Tris–HCl, pH 8.0, 2 mM dithiothreitol, 100 mM KCl, 5 mM MgCl2, and 10 µM oligo(dT), and cellular dNTP extracts (diluted to be within linear range of the assay, 2–50%) in a final volume of 20 µl/reaction. Reactions were incubated at 37 °C for 5 min and then quenched with 10 µl of 40 mM EDTA and 99% (vol/vol) formamide at 95 °C for 2 min The reactions were resolved on a

14% urea-PAGE gel (AmericanBio, Inc.) and analyzed using Pharos FX molecular imager (Biorad). The images were analyzed using ImageLab software.

4.12. Vpx and dNs treatment

Virus-like particles containing Vpx were generated as previously described (Hollenbaugh et al., 2014). PMA differentiated THP-1 were transduced with Vpx containing VLPs 18 h prior to infection with HIV-1 GFP. At 4 h prior to infection, cells were also treated with 2.5 mM dNs. Cells were subsequently washed with PBS twice and infected as described above.

4.13. Statistical analysis

Data were analyzed using the Student *T* test or Two-way ANOVA followed with Bonferroni test in Graphpad 5.0. Statistical significance was defined at $P<0.05$.

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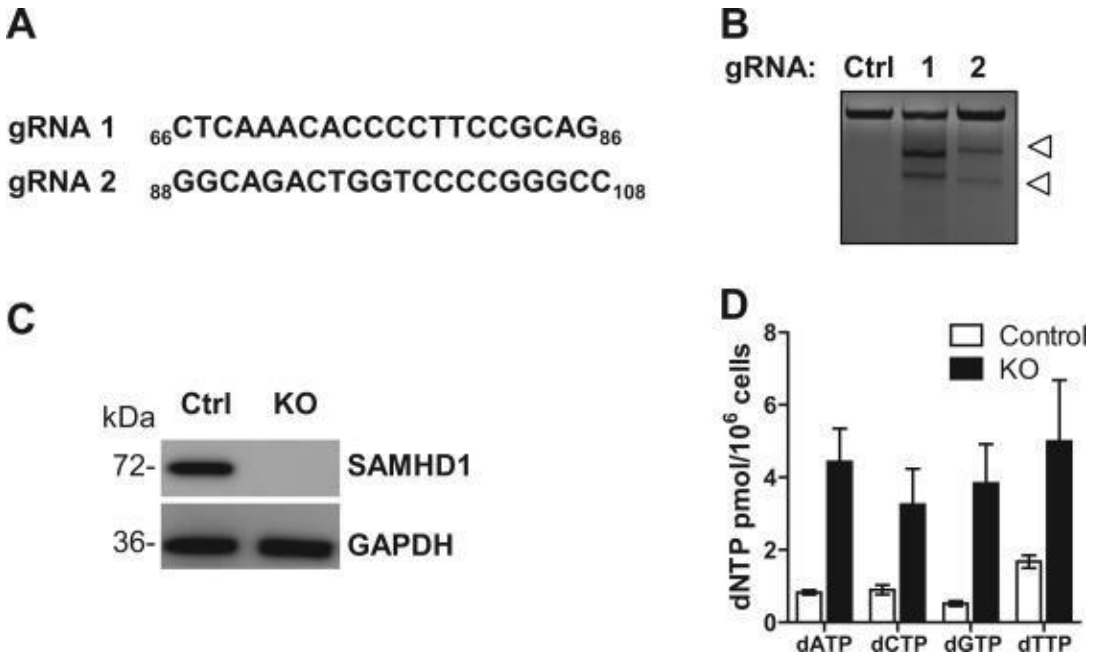


Fig. 1.

SAMHD1 knockout in THP-1 cells by CRISPR/Cas9. (A) CRISPR/Cas9 gRNA sequences used to produce the THP-1 SAMHD1 KO cells. Indicated sequences target the exon 1 of the *SAMHD1* gene (nucleotide number of exon 1 indicated as subscript). (B) Surveyor nuclease assay confirming SAMHD1 gRNA constructs. Genomic DNA was isolated from a stably transduced and selected polyclonal population of THP-1 cells, followed by PCR amplification of the *SAMHD1* targeted region. Amplicons were slowly reannealed. Surveyor nuclease-mediated cleavage of the heteroduplexes generated was detected in DNA from cells transfected with either gRNA 1 or 2 (white arrows), confirming that the gRNAs efficiently target*SAMHD1*. (C) SAMHD1 protein expression in non-differentiated THP-1 SAMHD1 control (clone 1) and KO cells (KO clone 1, derived from transduction with gRNA 2) confirms efficient knockout of the *SAMHD1*gene. GAPDH was used as loading control. (D) SAMHD1 KO cells show increased dNTP levels compared to control cells. Intracellular dNTP concentration in non-differentiated THP-1 cells expressing or not SAMHD1 was determined by single nucleotide incorporation assay. Error bars represent standard deviation of duplicate biological samples.

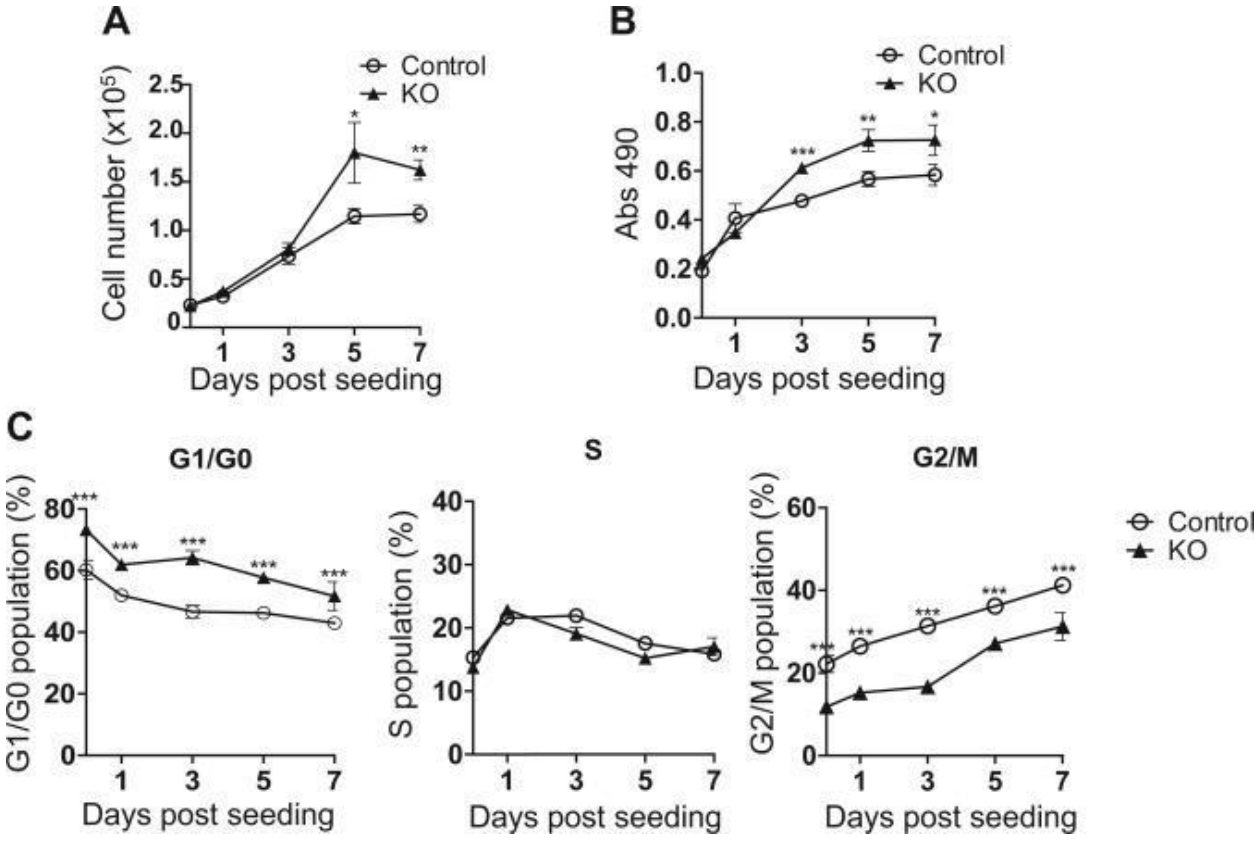


Figure 2. THP-1 SAMHD1 knockout cells have increased cell proliferation and altered cell cycle status. (A) Live cell counting. SAMHD1 control and KO cells were cultured for 7 days and, at the indicated time points, the number of viable cells was determined by trypan blue exclusion. Error bars represent standard deviation of quadruple samples. Statistical analysis was performed with the unpaired *T*-test with Welch's correction (**P*=0.0268,***P*=0.0011). (B) Proliferation of SAMHD1 control and KO cells was measured in time course by MTS assay. Error bars represent standard deviation of four replicates. Statistical analysis was performed using the unpaired *T*-test with Welch's correction (**P*=0.0130,***P*=0.0053,****P*=0.0002). (C) Alteration of the cell cycle by SAMHD1. Flow cytometry analysis of cell cycle progression in SAMHD1 KO cells in comparison to control cells was performed by propidium iodide staining. Error bars represent standard deviation of triplicate samples. Statistical analysis was performed using the Two-way ANOVA (***P*<0.01,****P*<0.001).

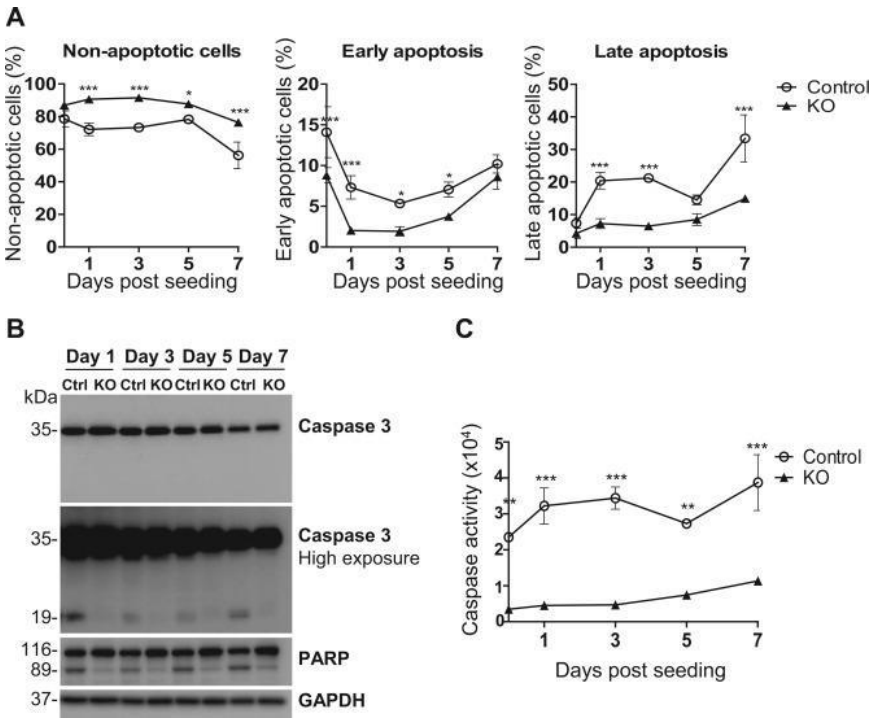


Fig. 3. Reduced apoptosis in SAMHD1 knockout THP-1 cells compared to control cells. (A) SAMHD1 control and KO cells were seeded in 12-well plate and, at day 0, 1, 3, 5 and 7, apoptosis was measured by flow cytometry via cell staining with PE-Annexin V (to detect phosphatidylserine exposure) and 7-AAD (to distinguish viable from non-viable cells). The left panel shows non-apoptotic, live cells (negative for Annexin V and 7-AAD staining); middle panel represents cells in early apoptosis, which are positive to Annexin V but negative to 7-AAD staining; the "Late apoptosis" panel on the right shows the percentage of cells exposing phosphatidylserine on the surface (positive for Annexin V) and with damaged cell membrane (positive for 7-AAD). Error bars indicate the standard deviation of triplicate samples. Statistical analysis was performed using the Two-way ANOVA (**P*<0.05,***P*<0.01,****P*<0.001). (B) Immunoblotting analysis of caspase 3 and PARP cleavage as markers of apoptosis induction. GAPDH was used as loading control. (C) Caspase 3/7 activities is significantly more pronounced in control cells compared to KO cells, as measured by a luminescent-based assay. Error bars represent standard deviation of two independent experiments, each performed in quadruplicates. Two-way ANOVA was performed for statistical analysis. (***P*<0.01,****P*<0.001).

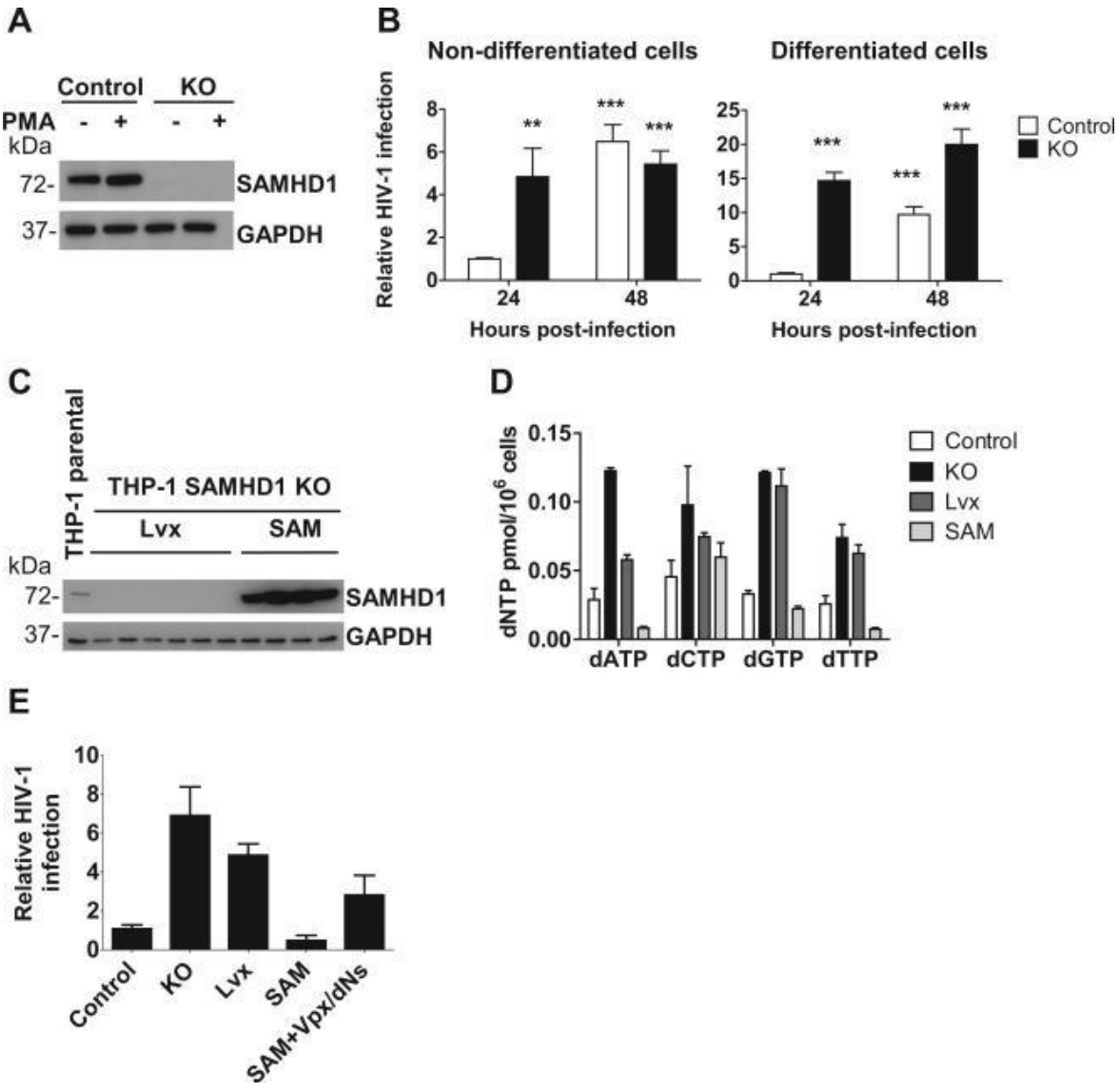
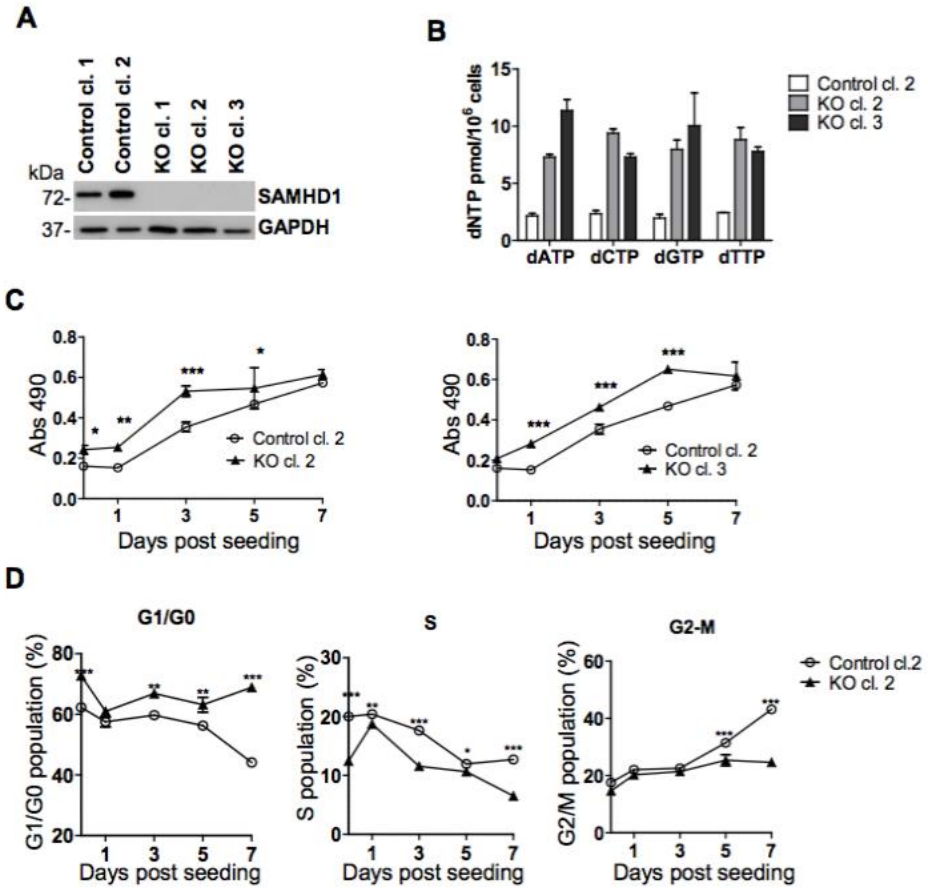
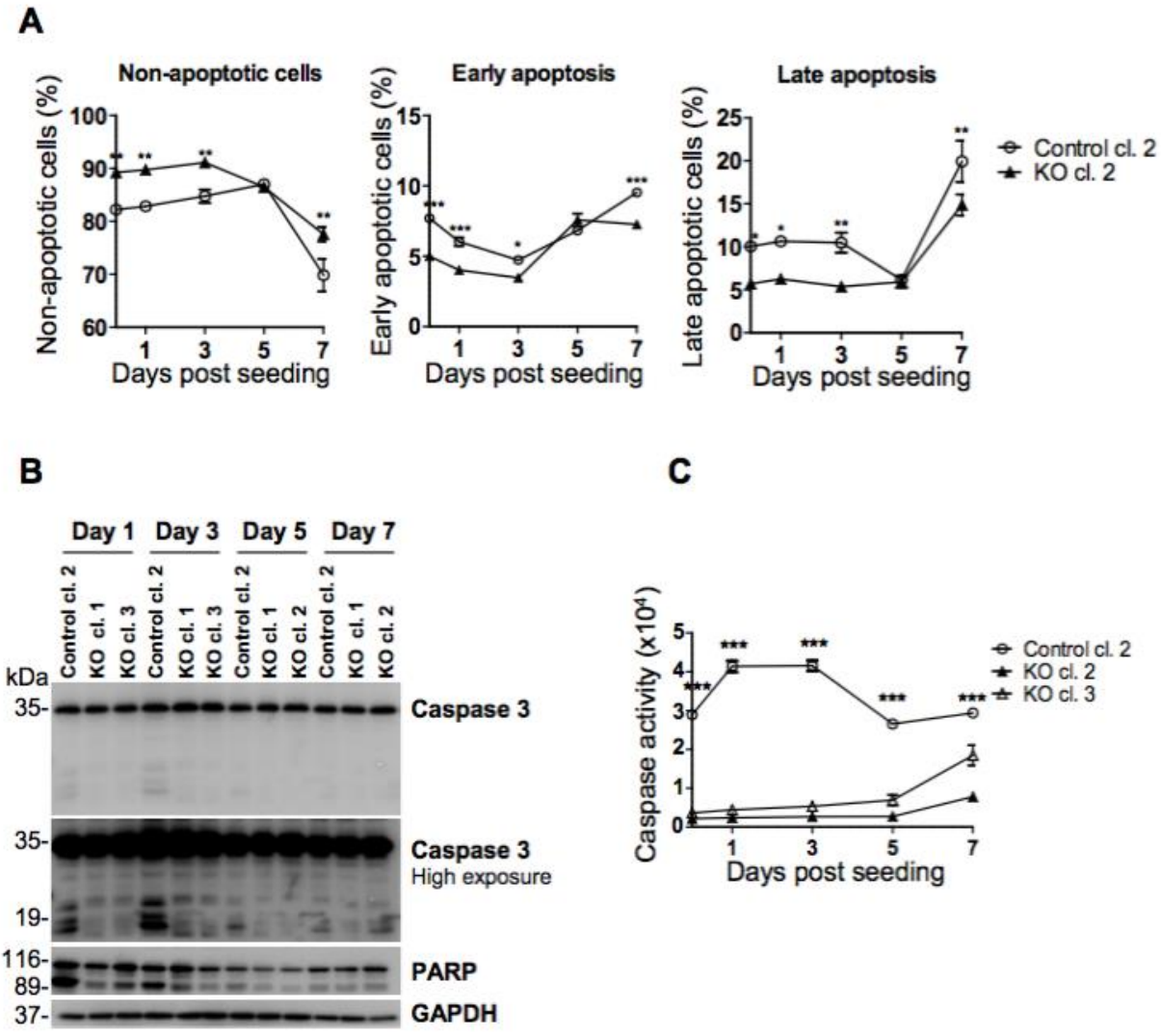


Fig. 4. Knockout of SAMHD1 increases HIV-1 infection of non-differentiated and differentiated THP-1 cells. (A) THP-1 SAMHD1 control and KO cells were grown in the absence or presence of 100 ng/ml of PMA for 24 h to induce differentiation into macrophage-like cells. Immunoblotting analysis using a specific SAMHD1 antibody confirmed efficient knockout of SAMHD1 in KO cells. (B) HIV-1 infection is increased in KO cells compared to control cells. Control and SAMHD1 KO cells, differentiated or not with PMA as described in (A), were infected with a single-cycle HIV-1-Luc/VSV-G at a multiplicity of infection (MOI) of 1. At 24 and 48 h post-infection, HIV-1 infection efficiency was measured by luciferase assay. Luciferase

values were normalized for protein concentration. The infection level in the control cells at 24 h post-infection was set as 1 and relative values are shown. A representative experiment performed with four replicates per sample is presented. Statistical analysis was performed with the unpaired *T*-test with Welch's correction (***P*=0.0019,****P*<0.0001). (C) SAMHD1 overexpression in KO cells. KO cells were transduced with LVX-IRES-mCherry control lentiviral vector (Lvx) or LVX-SAMHD1-IRES-mCherry vector (SAM). Cells were sorted by flow cytometry and SAMHD1 expression was analyzed in six Lvx and four SAM clones after differentiation with PMA by immunoblotting using a SAMHD1 specific antibody. GAPDH was used as loading control. (D) Rescue of SAMHD1 in KO cells restores SAMHD1-mediated depletion of dNTP levels. Intracellular dNTP concentration was measured in differentiated cells as described in Fig. 1(D). (E) Effect of SAMHD1 KO and overexpression on HIV-1 restriction. Cells were infected with a GFP-expressing HIV-1 virus and, after 72 h, the percentage of GFP-positive cells was measured by flow cytometry. While silencing of SAMHD1 results in more efficient HIV-1 infection, overexpression of SAMHD1 in KO cells restores SAMHD1 restriction effects. Counteraction of SAMHD1-mediated viral restriction was detected in SAM cells co-treated with Vpx and dNs.



Supplementary Fig. 1. Effects of SAMHD1 silencing on dNTP levels, cell proliferation and cell cycle progression of THP-1 cells. (A)Immunoblotting analysis confirmed efficient *SAMHD1* knockout in non-differentiated THP-1 SAMHD1 control and KO cell clones. GAPDH was used as loading control. (B)Intracellular dNTP levels in non-differentiated THP-1 control and SAMHD1 KO clones by single nucleotide incorporation assay. (C) MTS and (D) cell cycle analyses were performed as described in Fig. 2B-C in the indicated SAMHD1 control and KO clones. Control clones 1 and 2 were produced by transducing THP-1 parental cells with control CRISPR lentiviral vector. KO clone 1, derived from transduction with gRNA 2; KO clones 2 and 3 were derived from transduction with gRNA 1.



Supplementary Fig. 2. SAMHD1 induces spontaneous apoptosis in THP-1 cells. (A) Apoptosis detection by Annexin V/7-AAD double staining as described in Fig. 3A was performed in additional THP-1 SAMHD1 control and KO cell clones. (B) Caspase 3 activation and PARP cleavage detection by immunoblotting. GAPDH was used as loading control. (C)Activation of caspase 3/7 in SAMHD1 control and KO clones over a period of 7 days.

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CHAPTER VI: DISCUSSION

The HIV Epidemic

The HIV epidemic remains a threat in America and abroad. The chronic nature of the disease means that those who are infected, and unaware of their status, can unknowingly infect others. Barack Obama, the president of the United States, took initiative in 2010 to coordinate a national response to the HIV epidemic with three primary goals: 1) reducing the number of new HIV infections, 2) increasing access to health care and improving outcomes for people living with HIV and 3) reducing HIV-related health disparities. Reduction of new HIV infections involves multiple sub-goals including increased HIV testing, blood supply screening, expectant mother screening, needle exchanges to minimize infections from injection drug use and lastly advances in HIV therapies (152). The first four subsets involve interventions at the level of public health, which is of great importance. The latter, advancement of HIV therapy, is dependent on research and development by the scientific and pharmaceutical communities.

Nucleotide Regulation, Viruses & Cancer

Deoxyribonucleotides are essential for the DNA replication of all organisms and many viruses. The synthesis and degradation of cellular dNTPs are fine-tuned by host proteins, RNR and SAMHD1, respectively. The parallels of RNR and SAMHD1 regulation are uncanny. They both exist in their active form as tetramers and use two allosteric sites to tailor their activity (128,144). Seemingly both enzymes have evolved to use dATP as their master regulator for their reciprocal activities. dATP is the last to by synthesized by RNR and it acts as an inhibitor when concentrations get high enough to bind the a-site, for which dATP has low affiinity. On the other hand, dATP has the highest affinity of all four dNTPs for allosteric site 2 of SAMHD1, making it the most likely to activate tetramerization and dNTPase activity of the protein. dATP also has the weakest affinity for active site of SAMHD1 making it the slowest to be hydrolyzed. Maintenance

of dNTP homeostasis is complex and intertwined with other important cellular functions such as cell cycle, DNA damage repair and apoptosis (46,109,121). The strict regulation of these enzymes yields the correct balance of dNTPs, which is essential to maintain genomic integrity (144,145).

As the one of the controllers of dNTP homeostasis, RNR is considered an excellent therapeutic target for both viruses and cancers (146). Hydroxyurea has been used in cancer therapy for decades and functions as a radical scavenger that inactivates RNR though reduction of the tyrosyl radical on the R2 and p53R2 subunits (153). HIV studies in nondividing cell types, such as quiescent T cells, led to the hypothesis that dNTPs regulate cell permissiveness to HIV (154,155). Preliminary *in vitro* studies showed that treating actively dividing T cells with hydroxyurea, inhibited HIV replication and mimicked the natural restriction found in quiescent cells (156). Additionally, the decrease in cellular dNTPs gives a competitive edge to NRTIs through enhancement of NRTI phosphorylation and decreasing competition for the RT active site (157). The synergistic interaction of hydroxyurea and NRTIs led to multiple clinical trials, all of which showed sustained viral suppression. However, hydroxyurea is no longer clinically pursued because it was found to accentuate NRTI toxicity (158).

In Chapter II, we evaluate an FDA-approved anticancer agent, clofarabine, for anti-HIV activity. This purine analog is transported into cells, and due to high affinity for deoxycytidine kinase it is rapidly phosphorylated to its active form; clofarabine di- and tri-phosphate (-DP, -TP). Clofarabine-DP and –TP inhibit RNR by binding to the a-site of R1 and inducing hexamerization, similar to the regulation of RNR by high concentrations of dATP. RNR inhibition and the subsequent decrease in cellular dNTPs further potentiates the phosphorylation of clofarabine by removing the negative feedback that dCTP imposes on deoxycytidine kinase. Additionally, Clofarabine-TP is incorporated by some DNA polymerases, again potentiating its own incorporation by reduction of dNTPs. Incorporation of clofarabine induces delayed chain

termination and subsequently the cancer cells undergo apoptosis (159). Our studies indicate that clofarabine is a potent (nM) inhibitor of HIV replication in dividing and nondividing cells, with limited toxicity. In macrophages and T cells, clofarabine induced RNR inhibition as expected and depleted dNTPs in a manner that is signature of RNR inhibitors. Importantly, we found that clofarabine-TP can be incorporated by RT and induce delayed chain termination, with increased incorporation and inhibition at the low dNTP levels found in nondividing cells (160). This study highlights the idea that dNTP regulation can be targeted as a therapy for both cancer and HIV.

In 2011, understanding of dNTP regulation became more complete with the discovery of SAMHD1 as the hosts only known dNTPase. SAMHD1 dNTPase activity is responsible for viral restriction in nondividing myeloid cells and its degradation by HIV-2/SIV Vpx increases cell permissivity. HIV-1 does not encode Vpx, and its Vpr is not capable of SAMHD1 degradation, so in myeloid cells SAMHD1 maintains low dNTP concentrations and the virus is limited in its replication (118,119).

The hydroxyurea studies done in the early 1990s used actively dividing cells, which do not robustly express SAMHD1, and when treated with hydroxyurea, cellular dNTPs were reduced allowing for increased NRTI efficacy (157). In the case of myeloid cells, SAMHD1 limits dNTPs (similar to HU treatment) but it may also hydrolyze the NRTI-TPs rendering them unable to compete with dNTPs for viral inhibition. In Chapter IV, the role of SAMHD1 was investigated in terms of cell-type specific NRTI efficacy. Macrophages, which robustly express SAMHD1, were treated with Vpx in *trans* to induce degradation of SAMHD1, followed by NRTI treatment (with AZT, ABC, TDF or ddC), and infection with HIV. Vpx treatment significantly reduced NRTI efficacy. Activated CD4+ T cells, which express SAMHD1 but to a much lesser extent, showed a similar trend, however, it was not as profound likely due to the limited amount of SAMHD1 present and high dNTP concentrations. These results suggested that SAMHD1 mediates NRTI efficacy through regulation of dNTP concentrations. Additionally, as NRTI-TPs are structurally

analogous to dNTPs, the degradation of these molecules by SAMHD1 was evaluated. An HPLC-based assay indicated that SAMHD1 does not detectably hydrolyze AZT-TP or any of the four ddNTPs (161). Interestingly, clofarabine-TP is a substrate for SAMHD1, albeit to what extent still needs to be investigated (162). This suggests, that SAMHD1 degradation of NRTIs is not absolute and novel NRTIs should be tested for this susceptibility.

NRTI efficacy is intimately tied to the cellular dNTP environment and these data imply that HIV-2, which encodes Vpx to degrade SAMHD1, may be less responsive to NRTI treatment, specifically in macrophages (161). HAART regimens for HIV-2 are complicated because NNRTI and PIs have no or very limited potency. NRTIs, while seemingly active, are not sufficient to sustain depressed viremia (163). The interplay between SAMHD1 and Vpx may play a role in less-than robust treatment of HIV-2.

Cellular nucleotide environment varies depending on cell type, cell cycle status and necessity for DNA damage repair. Two cell types that are of great interest for HIV research are macrophages and activated CD4 T cells. Macrophages and activated CD4+ T cells have high levels of rNTPs, which are necessary for mRNA transcription, energy metabolism and signal transduction. Macrophages, which are nondividing, harbor very low dNTP concentrations (20-50 nM), in contrast to activated CD4+ T cells, which have high dNTP concentrations (2-5 μM) (43). The low dNTP pools in macrophages allows for the frequent incorporation of rNTPs by the error-prone HIV RT (164). This nuance of reverse transcription, found only in the nondividing cell dNTP environment, led to the hypothesis that ribonucleotide analogs could be used as cell-type specific HIV inhibitors (77,164-166).

In Chapter III, 5-azacytidine (5-aza-C) and 5-aza-2'-deoxycytidine (5-aza-dC) were evaluated for their inhibition of HIV replication by induction of lethal mutagenesis (76,77). These NRTIs purport their activity through promiscuous base pairing which leads error-ridden replication. RNA viruses, which replicate close to their error threshold, are particularly sensitive

to these molecules, which induce so many mistakes that the genome is no longer capable of producing viable progeny (75). Previously, 5-aza-C had been shown to have antiviral activity but the mechanism still remained unclear (77). Hypothetically, inhibition could be due to the direct incorporation of 5-aza-CTP into viral RNA, or 5-aza-CDP could be reduced by RNR and the product 5-aza-dCTP is incorporated in viral DNA or possibly both 5-aza-CTP and 5-aza-dCTP exert an antiviral effect.

Analysis of drug metabolites and Illumina deep sequencing indicated that 5-aza-C is rapidly reduced by RNR and 5-aza-dCTP is the species giving rise to the majority of the antiviral activity. In fact, in a follow-up study we showed that even in the presence of RNR inhibitors, 5-aza-C was still rapidly reduced to 5-aza-dCTP (166). One caveat of this study is that analyses were done in dividing cells, where RNR expression is robust. Biochemical analysis showed that RT is capable of incorporating 5-aza-CTP and 5-aza-dCTP. Potentially, in nondividing cells where RNR activity is at is lowest, 5-aza-CTP may be incorporated prior to reduction by RNR.

From a drug development standpoint, ribonucleoside analogs are attractive as antivirals because they are generally less complicated and cheaper to synthesize. Additionally, potent ribonucleoside analogs that are active against HIV may be used broadly against other RNA viruses. However, the findings of this study suggest that the efficiency of RNR reduction of investigatory ribonucleoside analogs should be examined. Ribavirin, a clinically available ribonucleoside analog, is used to treat RSV and hepatitis C. It's potency against RNA viruses is attributed to incorporation into viral RNA, which leads to hypermutation or lethal mutagenesis (167). Interestingly, ribavirin also inhibits DNA viruses although its reduced deoxyribose analog is completely inactive. It would be of great mechanistic interest to investigate whether the 2' deoxyribose ribavirin is an inhibitor of RNR, which would account for its restriction of DNA viruses despite the lack of antiviral potency of 2' deoxyribose ribavirin (168).

Many cancers therapies rely on the inhibition of RNR in order to starve the fast growing cancer cells of nucleotides. However, studies with clofarabine have shown, some nucleoside analogs can be degraded by SAMHD1, potentially reducing efficacy (162). It has been purported that SAMHD1 may be an interesting target for cancer therapies (145). Although possible, the necessity to increase SAMHD1 dNTPase activity will be more difficult to target therapeutically than the inhibition of RNR. Further definition of the dNTP regulation pathways, both catabolic and anabolic, will better inform the potential exploits for cancer (and viral) treatment.

In chapter V, the physiological role of SAMHD1, specifically in cell cycle and apoptosis, are investigated. These studies provided a unique obstacle in that; SAMHD1 is most highly expressed in nondividing cells. Therefore, to study the effects of SAMHD1 on cell cycle we created a CRISPR/Cas9 KO SAMHD1 cell line in THP-1 cells, a dividing monocytic cell line that can be differentiated to nondividing macrophage-like cells. The SAMHD1 KO cells had increased dNTPs and relieved HIV restriction, which was the expected phenotype. Alteration of cell cycle progression was also observed, with SAMHD1 KO cells having increased proliferation, metabolism indicating that SAMHD1 negatively regulated cell cycle progression. Furthermore, silencing of SAMHD1 increased resistance to apoptosis. Taken together these data suggest that regulation SAMHD1, similar to RNR, is intertwined with cell cycle and apoptosis. The implications for cancer are clear, losing the capability to regulate dNTPs (through SAMHD1 or RNR) cause aberrations to cell cycle and sensitivity to apoptosis (121).

Insight into the cell's regulation of dNTPs provides us with invaluable data for the development of therapeutics for both viruses and cancer. Therapeutic innovation is essential due to the increased prevalence of HIV NRTI resistance, transmission of drug resistant variants and necessity of salvage therapy. Ribonucleoside analogs, for treatment of RNA viruses and potentially as a cell-type specific HIV drug, need to be evaluated for ability to be reduced by RNR and if they are reduced the efficacy of the deoxyribose form must be considered. NRTIs,

whether they are the classic obligate chain terminator, delayed chain terminator, or lethal mutagen should be screened for the following characteristics: 1) Inhibition of RNR, 2) Inhibition of HIV-RT, including NRTI resistant variants and 3) Resistance to degradation by SAMHD1, 4) Toxicity in conjunction with HAART regimens.

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